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Award Number: W81XWH-17-1-0398

TITLE: Simultaneous Ligand-Directed Cytotoxic Toxin and Endosome Disruptor Delivery to Ablate Prostate Cancer

PRINCIPAL INVESTIGATOR: Josef Vagner

CONTRACTING ORGANIZATION: University of Arizona
Tucson, AZ 85719

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Simultaneous Ligand-Directed Cytotoxic Toxin and Endosome Disruptor Delivery to Ablate Prostate Cancer

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6. AUTHOR(S)
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5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Arizona
PO Box 210158, Rm 510
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University of Georgia

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U.S. Army Medical Research and Materiel Command
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12. DISTRIBUTION / AVAILABILITY STATEMENT

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this grant we proposed to develop toxins that targeted GnRH-R and endosome disruptors that targeted bombesin 2 receptors to treat prostate cancer. In this first year, we have made GnRH-Gelonin and GnRH-Saporin conjugates. We have also established that GnRH-Gelonin and GnRH-Saporin conjugates induce apoptosis in a metastatic human prostate cancer cell lines (PC-3). We have further shown that targeting the endosome disruptor, listeriolysin O (LLO), to PC-3 cells using the kisspeptin receptor agonist (metastin) induces apoptosis. Metastin targeted LLO increases apoptosis induced by GnRH-Gelonin and/or GnRH-Saporin conjugates. We have developed a 2-step FPLC based purification for a his-tagged LLO that includes hydrophobic interaction chromatography followed by nickel affinity chromatography. We have also developed a 2-step FPLC based purification of his-tagged Gelonin purification, which includes nickel affinity chromatography followed by size exclusion chromatography. We have synthesized D-Lys6 GnRH and the Bombesin 2 receptor agonist, D-Phe$_6$-Nle$_{14}$ Bombesin7-14. These will be conjugated to LLO for additional *in vitro* tests. As proposed in the initial application, i*n vivo* model development and testing will begin this year and will take full advantage of current and future *in vitro* results.

15. SUBJECT TERMS
Prostate Cancer, Targeted Toxin,

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. **INTRODUCTION:** Prostate cancer is the 2nd most common cancer, representing 10.7% of all new cancer cases within the U.S [6]. Prostate cancer will result in the death of 26,120 men in the U.S., making it the 2nd leading cause of cancer death in men. With an estimated 2.8 million men living with prostate cancer in the U.S. (2013), a safe and effective therapeutic option would relieve the burden of this disease on our health care system. The most common treatments for prostate cancer are surgical prostatectomy or radiation therapy. However, these two treatment options are only viable for early stage prostate cancers. Prostate cancers that have metastasized are most commonly treated with androgen depletion therapy. Androgen depletion therapy is transiently effective, with progression recurring after 18 months (median). Thus, there remains a demand for an effective treatment to combat metastatic prostate cancer. **S**ince chemotherapeutics are preferred for metastatic cancers, an effective systemic treatment would provide an option for patients with metastatic prostate cancer, a very deadly disease with a 5-year survival rate of only 28%. In our grant, we proposed 3 aims that will optimize the *efficacy* of GnRH targeted toxins toward human prostate cancer cell lines in vitro and in a mouse xenograft model of metastatic human prostate cancer *in vivo.* We are targeting the Gonadotropin releasing hormone (GnRH) and Bombesin 2 (BB2) receptors to simultaneously deliver toxin and an endosome disruptor to prostate cancer cells. Since only prostate cancer cells express both the GnRH and BB2 receptors, our targeting strategy using these 2 receptors is specific to prostate cancer cells. Although, similar strategies may be applied to target any cancer and therefore our work has a cancer-wide scope.

2. **KEYWORDS:** Prostate Cancer, Targeted Therapeutics, GnRH, GnRH Receptor, Bombesin, Gastrin Releasing Peptide, Endosome, Lysosome, PC-3, Gelonin, Saporin, Listeriolysin O, Doxorubicin, Zoptarelin Doxorubicin

3. **ACCOMPLISHMENTS:** We have primary focused on 1) Purification procedures Gelonin and Listeriolysin O synthesis in E.coli, 2) Synthesis of GnRH-Gelonin and GnRH-Saporin conjugates, 3) testing for inhibition of protein synthesis by Gelonin conjguates and lysis of red blood cells by Listeriolysin O conjugates, and 4) Testing *in vitro* apoptosis efficacy in PC-3 human prostate cancer cells. We have established that GnRH-Gelonin and GnRH-Saporin conjugates can effectively induce apoptosis in PC-3 cells. We have observed that GnRH-Saporin conjugates are more effective than Gelonin. We have further shown that including a targeted endosome disruptor improves efficacy. We expect to synthesize the BB2 and GnRH targeted Listeriolysin O conjugates in the next few months. We further have plans to synthesize a GnRH-Saporin conjugate with 4-5 GnRH molecules per molecule of Saporin. This will differ from the current conjugate which has on average 1.8 GnRH molecules per molecule of Saporin. By increasing the number of GnRH molecules conjugated to Saporin we expect to increase the avidity of the conjugate for the target prostate cancer cells. We have met all stated goals to date. Below we address each specific aim, major task, and specific task in detail.

Specific Aim 1: Develop GRP-R and GnRH-R agonist conjugates with the endosome disrupter, listeriolysin O (LLO), the ribosome inactivating protein-gelonin (toxin), and doxorubicin a toxin that preferentially targets mitotic cells.

Major Task 1: Synthesis and linker optimization of GnRH- and GRP- LLO, Gelonin, and doxorubicin conjugates

Timeline: Year 1 Quarter 1 – Year 2 Quarter 1

% Completion: 75%

Research Site: Vagner Laboratory – University of Arizona

Work toward accomplishing this task: GnRH and D-Phe6-Nle14 Bombesin7-14, a BB2 agonist, have been synthesized. GnRH-Gelonin and GnRH-Saporin conjugates have been made. We are currently altering the number of GnRH molecules on Gelonin and Saporin to further improve efficacy. We intend to complete the first synthesis of LLO conjugates with GnRH/ BB2 ligands and GnRH-Doxorubicin in the next quarter. The synthesis of GnRH and BB2 ligands are carried out using solid-phase method. Structures were selected to balance binding affinity of ligand to the cognate receptor and its stability. GnRH analog requires both C and N termini intact as pyroglutamate and amide, respectively. Therefore, we have chosen side chain of D-Lys6 as an attachment point to proteins. Peptide was derivatized in this position with 3-methyl-3-mercaptopropionic acid and released from solid-phase resin as a free thiol. The thiol group was activated with 2-thiopyridine activator then reacts with free thiols of protein to form Saporin/gelonin GnRH conjugates. The proteins were overpopulated with unnatural thiol groups using amine-reacting Traut's reagent or it's methylated analog then the excees of Traut's reagent was removed by size exclusion chromatography. The final products were purified using the size exclusion chromatography and characterized by HPLC and MS.

Subtask 1: Test LLO conjugate hemolysis activity

Timeline: Year 1, Quarter 1 – Year 2, Quarter 1

% Completion: 25%

Research Site: Renquist Laboratory – University of Arizona

Work toward accomplishing this task: We have tested hemolysis activity of purified LLO and have a pure LLO that can be used for synthesis of the LLO conjugates.

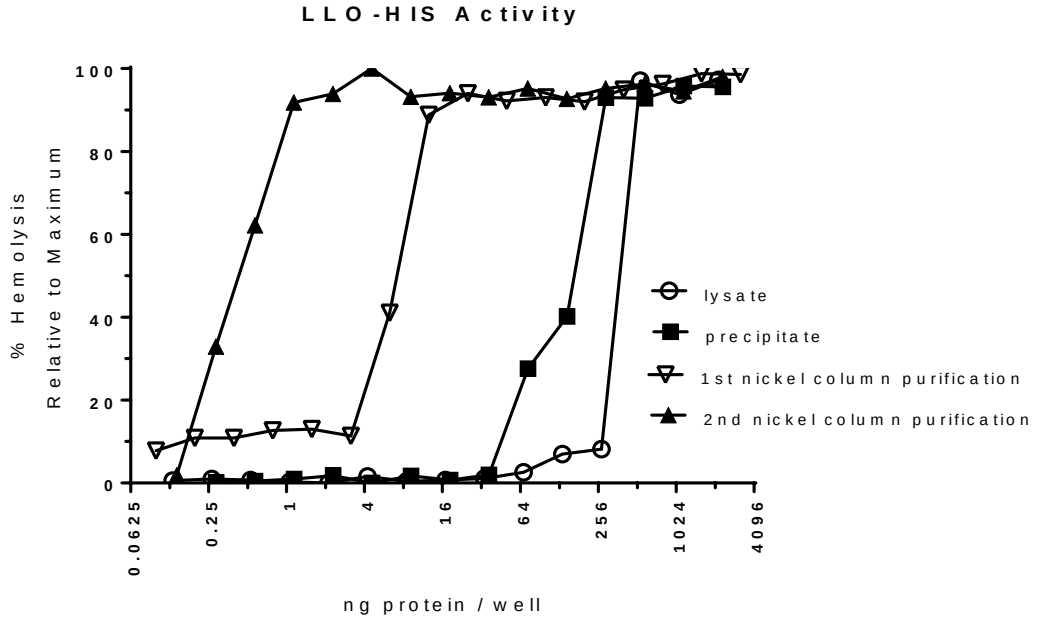

Figure 1. We have purified E.Coli produced 6X his-tagged Listeriolysin O (LLO-His), as shown by the improved hemolytic potential of the proteins after Ammonium Sulfate precipitation and 2 runs through a nickel column with a gradient increase in imidazole. Units are ng protein/well (200 ul total volume).

Subtask 2: Test Gelonin conjugate protein synthesis inhibition.

Timeline: Year 1, Quarter 1 – Year 2, Quarter 1

% Completion: 100%

Research Site: Renquist Laboratory – University of Arizona

Work toward accomplishing this task: We have shown that the GnRH-Gelonin and GnRH-Saporin conjugates with 1-2 GnRH molecules inhibit protein synthesis similarly to Gelonin or Saporin alone. We will continue to test additional Gelonin and Saporin conjugates.

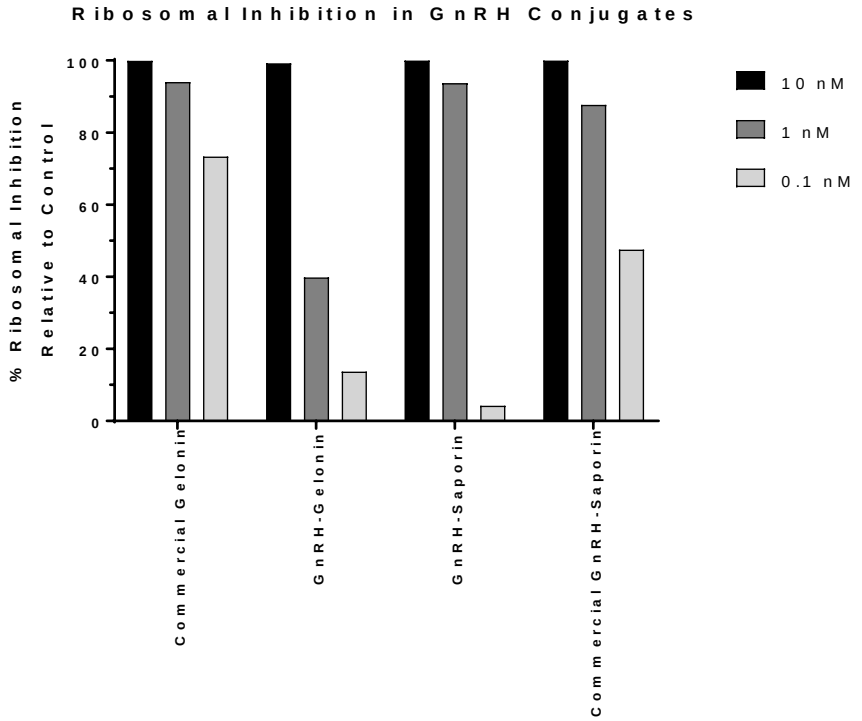

Figure 2. Inhibition of protein synthesis by Gelonin, GnRH-Gelonin conjugate synthesized in the Vagner laboratory, GnRH-Saporin conjugates synthesized in the Vagner Laboratory, or GnRH-Saporin conjugates made by Advanced Targeting Systems (Cat. # Beta-028; San Diego, CA). This test was performed using the Promega rabbit reticulocyte lysate assay (Cat. # L4960; Madison, WI)

Major Task 2: Synthesize sufficient quantities of optimized conjugates for in vivo testing
Timeline: Year 2, Quarter 2 – Year 3, Quarter 4
% Completion: 5%
Research Site (s): Vagner and Renquist Laboratories – University of Arizona
Work toward accomplishing this task: We have worked to develop methods to purify large quantities of Listeriolysin O from E. Coli.

Subtask 1: Continue additional optimizations of linker
Timeline: Year 2, Quarter 2 – Year 3, Quarter 4
% Completion: 25%
Research Site(s): Vagner, Renquist, and Selvaraj Laboratories
Work toward accomplishing this task: We are actively optimizing the number of GnRH molecules on Gelonin and Saporin conjugates.

Specific Aim 2: Perform in vitro testing on two human prostate cancer cell lines (PC-3 and MDA-PCa-2b) to optimize the combination of ligand-toxin and ligand-endosome disrupter to target multiple prostate cancers.

Major Task 1: In vitro tests of efficacy to optimize the linker for conjugates of BB2 and GnRH-R agonist to payload toxins (doxorubicin and gelonin) and LLO.

Timeline: Year 1, Quarter 1 – Year 3, Quarter 4

% Completion: 25%

Research Site: Selvaraj Laboratory – University of Georgia

Work toward accomplishing this task: We have tested the efficacy of GnRH-Saporin and GnRH-Gelonin in PC-3 cells. We have shown that both of these conjugates are effective (Figure 4, below).

Subtask 1: Ensure specificity to only those cells that express target receptors by using cells that express only BB2, only GnRH-R or neither receptor.

Timeline: Year 1, Quarte 1 – Year 3, Quarter 4

% Completion: 0%, We have not set out to establish specificity yet.

Research Site: Selvaraj Laboratory – University of Georgia

Major Task 2: Identify the maximum concentration of ligand-directed endosome disruptor that in not cytotoxic for each cell line.

Timeline: Year 2, Quarter 1

% Completion: 25%

Research Site: Selvaraj Laboratory – University of Georgia

Work toward accomplishing this task: We have established the maximum concentration of metastin targeted LLO that is not cytotoxic. We will continue this for all LLO conjugates. Accordingly, this timeline should be extended to include the entire time from Year 2, Quarter 1 – Year 3, Quarter 4.

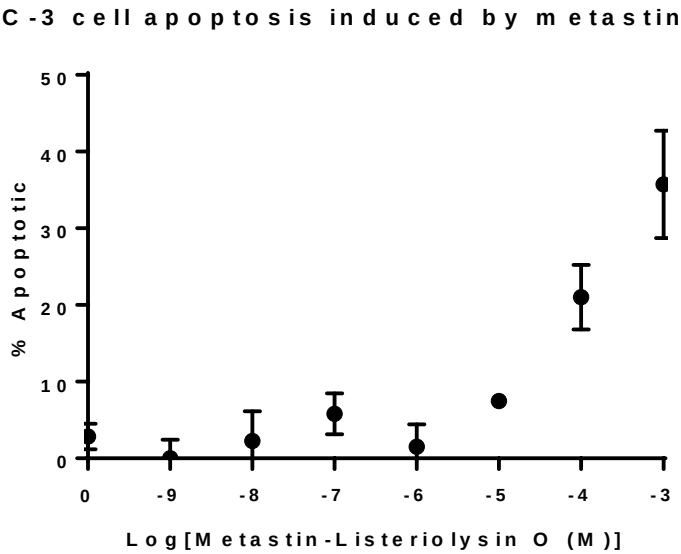

Figure 3. Metastin targeted LLO induces apoptosis in PC-3 cells beginning at 100 µM. PC-3 cells were exposed to compounds for 48 hours and % apoptosis was assessed by flow cytometry using the annexin V assay.

Subtask 1: Ensure specificity to only those cells that express target receptors by using cells that express only BB2, only GnRH-R or neither receptor.

Timeline: Year 2, Quarter 1

% Completion: 0%, We intend to do this for all LLO conjugates. Accordingly, this timeline should be extended to include the entire time from Year 2, Quarter 1 – Year 3, Quarter 4.

Research Site: Selvaraj Laboratory – University of Georgia

Major Task 3: Find the minimum effective concentration of ligand directed toxins.

Timeline: Year 2, Quarter 2 – Year 2, Quarter 3

% Completion: 25%

Research Site: Selvaraj Laboratory – University of Georgia

Work toward accomplishing this task: We have performed dose responses for the GnRH-Gelonin and GnRH-Saporin conjugates in PC-3 cell lines (See Figure 4, below). We will continue to perform these studies with all future conjugates and in the MDA-PCa-2b cells.

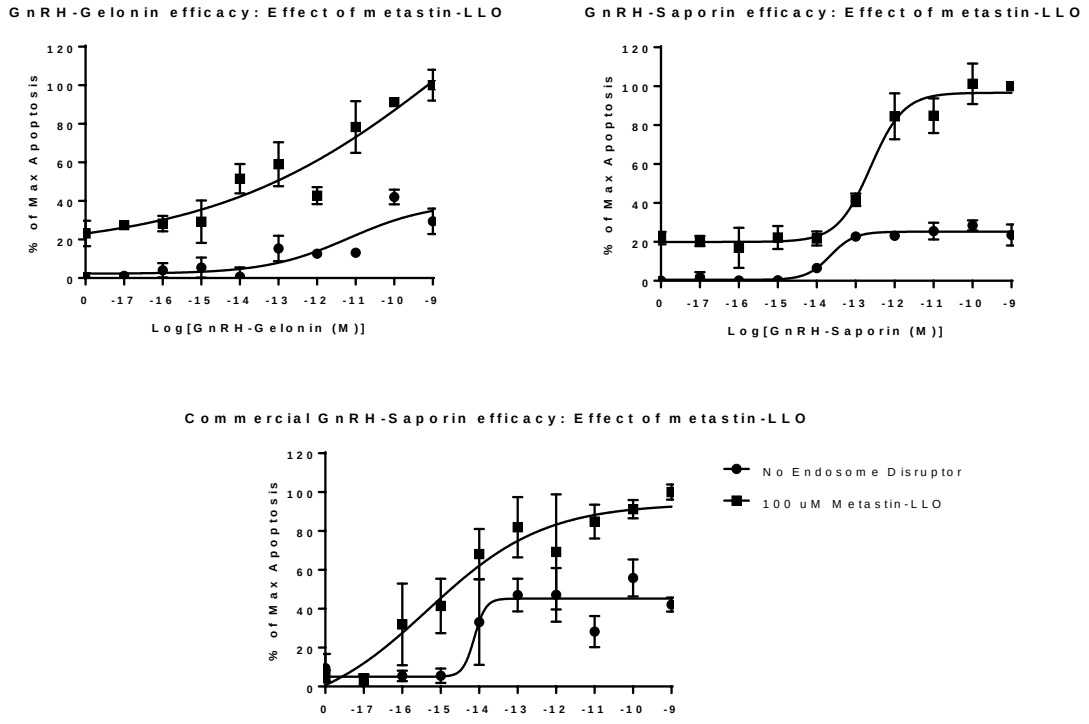

Figure 4. GnRH-Ribosome inactivating conjugate efficacy (measured as ability to induce apoptosis) is enhanced by co-incubation with metastin-LLO. This improvement in efficacy is more robust when metastin-LLO is provided at 100 µM than at 10 µM. It does appear that GnRH-Saporin conjugates are more effective. Accordingly, we intend to focus on Saporin

conjugates. PC-3 cells were exposed to compounds for 48 hours and % apoptosis was assessed by flow cytometryusing the annexin V assay.

Subtask 1: Ensure specificity to only those cells that express target receptors by using cells that express only BB2, only GnRH-R or neither receptor.

Timeline: Year 2, Quarter 2 – Year 2, Quarter 3

% Completion: 0%, we will perform these tests as described.

Research Site: Selvaraj Laboratory – University of Georgia

Major Task 4: Identify the minimum concentration of ligand directed LLO that maximally encourages ligand directed toxin efficacy.

Timeline: Year 2, Quarter 2 – Year 2, Quarter 3

% Completion: 10%

Research Site: Selvaraj Laboratory – University of Georgia

Work toward accomplishing this task: We have tested 2 doses of metastin directed LLO and have shown that the efficacy of metastin LLO drops as the concentration decreases from 100 µM to 10 µM (Figure 5).

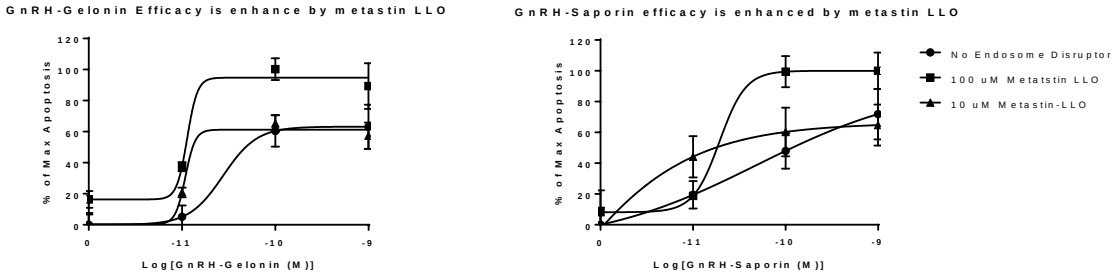

Figure 5. GnRH-Ribosome inactivating conjugate efficacy (measured as ability to induce apoptosis) is enhanced by co-incubation with metastin-LLO. This improvement in efficacy is more robust when metastin-LLO is provided at 100 µM than at 10 µM. PC-3 cells were exposed to compounds for 48 hours and % apoptosis was assessed by flow cytometry using the annexin V assay.

Subtask 1: Ensure specificity to only those cells that express target receptors by using cells that express only BB2, only GnRH-R or neither receptor.

Timeline: Year 2, Quarter 2 – Year 2, Quarter 3

% Completion: 0%

Research Site: Selvaraj Laboratory – University of Georgia

Work toward accomplishing this task: None

Specific Aim 3: Perform in vivo testing of the antitumor effects of our combined targeted toxin/targeted endosome disruptor treatments in mice bearing xenografts of the MDA-PCa-2b and PC-3 prostate cancer cell line

Major Task 1: Develop and Optimize the In vivo Models. Including donor mice
Timeline: Year 1, Quarter 4 – Year 2, Quarter 1
% Completion: 0%
Research Site: Renquist Laboratory – University of Arizona
Work toward accomplishing this task: We intend to begin studies in mice in the next month.

Major Task 2: Assess In vivo Efficacy of Targeted Endosome Disruptors and Targeted Toxins.
Timeline: Year 2, Quarter 2 – Year 2, Quarter 4
% Completion: 0%
Research Site: Renquist Laboratory – University of Arizona
Work toward accomplishing this task: N/A
Subtask 1: Assess Efficacy
Subtask 2: Assess Toxicity

Major Task 4: Optimization of a Treatment Paradigm to Eliminate Prostate Cancer Tumor Burden.
Timeline: Year 3, Quarter 1 – Year 3, Quarter 4
% Completion: 0%
Research Site: Renquist Laboratory – University of Arizona
Work toward accomplishing this task: N/A
Subtask 1: Assess Efficacy
Subtask 2: Assess Toxicity
Subtask 3: Address Tumor Elimination MRI/PET

- **What opportunities for training and professional development has the project provided?**
  - *Nothing to Report*
- **How were the results disseminated to communities of interest?**
  - *Nothing to Report. We do intend to submit at least 1 manuscript in the next year.*
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - *Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - In the first year we have shown that targeting an endosome disruptor to a cancer cell improves the efficacy of targeted toxins.
- **What was the impact on other disciplines?**
  - *Nothing to Report*
- **What was the impact on technology transfer?**
  - *Nothing to Report. We had previously disclosed this invention and we expect that this will be advanced toward provisional patent protection prior to the conclusion of this work.*
- **What was the impact on society beyond science and technology?**
  - *Nothing to Report. However, this work has the potential to improve the application of targeted therapeutics.*

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - *None*
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - We have had difficulty in purifying sufficient amounts of Listeriolysin O. The initial problem was due to cleaving off of the C-terminal 6X his tag. We solved this issue by re-creating the plasmid to include an N-terminal 6X his tag instead of a C-terminal his tag. Our second issue involved optimizing the purification of the Listeriolysin O product. We have switched from gravity flow columns to FPLC purification and have used an imidazole gradient to improve purity. Having cleared this hurdle, we expect to be able to rapidly synthesize LLO conjugates for *in vitro* and *in vivo tests.*
- **Changes that had a significant impact on expenditures**
  - The Renquist lab expenditures have been less than expected as we are just now initiating *in vivo* testing. As studies move forward expenditures by the Renquist lab are expected to increase.
  - Dr. Selvaraj has spent approximately 60% of the allotted budget for the calendar year 2018.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - *None*

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

    - **Publications, conference papers, and presentations**
      Report only the major publication(s) resulting from the work under this award.
        - **Journal publications.** *Nothing to Report*
        - **Books or other non-periodical, one-time publications.** *Nothing to Report*
        - **Other publications, conference papers, and presentations.** *Nothing to Report*
    - **Website(s) or other Internet site(s)**
      Nothing to Report
    - **Technologies or techniques**
      Nothing to Report
    - **Inventions, patent applications, and/or licenses**
      Nothing to Report
    - **Other Products**
      Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	*Benjamin Renquist*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	0000-0003-1517-8226
Nearest person month worked:	2
Contribution to Project:	I have directed the purification of Listeriolysin O and Gelonin and the tests of LLO and Gelonin Efficacy. This involved a good deal of problem solving and evaluating the literature.
Funding Support:	

Name:	*Kyle Kentch*
Project Role:	*Research Staff*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Kyle led the purification of Listeriolysin O and Gelonin and conducted tests of LLO and Gelonin Efficacy.
Funding Support:	Found Animals Foundation. They funded research that is using Gelonin and LLO conjugates for the development of an injectable sterilant.

Name:	Ramesh Selvaraj
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	I have conducted the in vitro efficiency of conjugates on PC3 cell lines. This involved a good deal of problem solving and evaluating the literature.
Funding Support:	

Name:	Tejit Pothuraju
Project Role:	*Undergraduate Student researcher*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Tejit conducted the in vitro efficiency of conjugates on PC3 cell lines. This involved learning flow cytometry techniques and cell culture techniques.
Funding Support:	

Name:	Keila Acevedo
Project Role:	*Graduate Student researcher*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Keila conducted the in vitro efficiency of conjugates on PC3 cell lines. This involved learning flow cytometry techniques and cell culture techniques.
Funding Support:	

Name:	Bailey Lester
Project Role:	*Graduate Student researcher*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Bailey conducted the in vitro efficiency of conjugates on PC3 cell lines. This involved learning flow cytometry techniques and cell culture techniques.
Funding Support:	

Name:	Josef Vagner
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	0000-0003-1289-0234
Nearest person month worked:	3
Contribution to Project:	I have designed and conducted pilot synthesis of Gelonin and Saporin conjugates with GnRH, solid-phase synthesis of GnRH and BB2 ligands, and conjugation chemistry based on sterically hindered disulphide bonds.
Funding Support:	Found Animals Foundation. They funded research that is using Gelonin and LLO conjugates for the development of an injectable sterilant.

Name:	Zhenyu Zhang
Project Role:	*Research Specialist*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Zhenyu performed the synthesis of conjugates, solid-phase synthesis of GnRH and BB2, their purification and analysis
Funding Support:	Found Animals Foundation. They funded research that is using Gelonin and LLO conjugates for the development of an injectable sterilant.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Renquist: No effect of funding changes on percent effort dedicated to this project

- New Active Funding:
  - **Arizona Biomedical Research Commission**
    Title: Targeting the Cause of Type II Diabetes Mellitus
    Dates: 06/01/2018-05/31/2021
    Role: Principal Investigator

- Completed Funding:
  - **American Heart Association: Beginning Grant In Aid**
    Title: The role of hepatocyte membrane potential in regulating arterial pressure
    Grant: 15BGIA25090300
    Dates: 7/1/2015-6/31/2018
    Role: Prinicpal Investigator
  - **Arizona Biomedical Research Commission Early Stage Investigator Award**
    Title: Targetting the Hepatocyte/Vagal Nerve Communication to Develop Therapeutics for Type 2 Diabetes
    Grant: ADHS14-082986
    Dates: 09/31/2014-10/01/2017
    Role: Principal Investigator

Selvaraj:

- Nothing to report

Vagner: No effect of funding changes on percent effort dedicated to this project

- New Active Funding:
  - **National Institute of Health**
    Title: Development of PAR2 Antagonists for Control of Asthma
    Grant: R21 AI140257
    Dates: 06/01/18-05/31/20
    Role: co-Principal Investigator
- Completed Funding:
  - **Found Animals Foundation**
    Title: Enhancing the Toxicity of GnRH- and Multivalent Targeted RIP Conjugates to Induce Sterility
    Grant: LTR DTD 10/21/15
    Dates: 10/1/15-9/30/17
    Role: co-investigator

- **What other organizations were involved as partners?**
  - *Nothing to Report*

8. **SPECIAL REPORTING REQUIREMENTS**

   - **COLLABORATIVE AWARDS:** *All PIs will submit this report to https://ers.amedd.army.mil for each unique award.*

9. **APPENDICES:** *None*