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

PRINCIPAL INVESTIGATOR: Ramesh Selvaraj

CONTRACTING ORGANIZATION: University of Georgia, Athens, GA

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14. ABSTRACT We proposed that the efficacy of G-protein coupled receptor (GPCR) ligand directed toxins is limited by sequestration and degradation in the endosome following endocytosis. We proposed that a GPCR ligand-endosome disruptor conjugate would enhance the efficacy of a GPCR ligand-toxin conjugate. We engineered E. Coli that synthesize listeriolysin O (LLO), an endosome disruptor, and developed a fast protein liquid chromatography method to purify this LLO. We used solid state protein chemistry to synthesize gastrin release peptide (GRP) and gonadotropin releasing hormone (GnRH) then created conjugates with the ribosome inactivating protein toxin, Saporin, and the endosome disruptor, LLO. Both GRP-Saporin and GnRH-Saporin were effective in ablating two immortalized prostate cancer cell lines (DU145 and PC3 cells) in vitro, with GRP-Saporin effective at lower concentrations. The efficacy of both GRP-Saporin and GnRH-Saporin were enhanced by simultaneous treatment with LLO conjugates respectively. This year we developed methods to effectively isolate mg quantities of LLO and make mass batches of LLO conjugates. We further established our mouse model of prostate cancer and tumor transplantation and treated pilot mice with our GRP-Saporin/GnRH-LLO combination treatment. We are currently conducting research to complete in vivo mouse studies aimed at understanding the efficacy of these conjugates in a tumor implant model. These in vivo studies will include a GPCR ligand-doxorubicin conjugate to test the efficacy of endosome disruptors in preventing loss of efficacy due to endosomal sequestration. By including doxorubicin conjugates our findings may be more quickly applied to improve a compound in phase 3 clinical trials.					
15. SUBJECT TERMS Prostate Cancer, Targeted Toxin, Doxorubicin, GnRH, GRP, Listeriolysin O					
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1. INTRODUCTION:

Our research aims to improve chemotherapeutic treatments for prostate cancer by improving the efficacy of ligand targeted toxins. Cancers overexpress G-protein coupled receptors, which are endocytosed upon ligand binding. In turn, GPCR ligand-toxin conjugates have been a focus of study in the cancer therapeutic field. However, efficacy has been limited, as endocytosis into the cell does not lead to cytoplasmic toxin delivery, but instead sequestration and/or degradation in the endosome. To encourage efficacy of ligand directed toxins we proposed to simultaneously deliver a ligand directed endosome disruptor. Our hypothesis was that this ligand directed endosome disruptor would be endocytosed with the ligand directed toxin, breaking out the toxin and encouraging efficacy. Our research aims involve 1) the synthesis of ligand directed toxins and ligand directed endosome disruptors, 2) *in vitro* tests of efficacy in 2 immortalized prostate cancer cell lines, and 3) *in vivo* tests of efficacy in mouse tumor models that apply these same cancer cell lines. We have created all of the conjugates, established dramatic improvements in efficacy *in vitro*, optimized the conjugate combination, and collected promising *in vivo* data showing that high dose GRP-Saporin/GnRH-LLO co-treatment stops tumor progression. We have found that although Listeriolysin-O (LLO) is a highly potent endosome disruptor, chemistry with this protein is difficult, and consistently creating a targeted LLO that maintains activity is problematic. Future research should work on identifying alternative endosome disruptors.

2. **KEYWORDS:** Prostate cancer, Ribosome inactivating protein, Saporin, Doxorubicin, Gonadotropin Releasing Hormone, GnRH, Gastrin Releasing Peptide, GRP, Bombesin 2 Receptor, Listeriolysin O, Targeted toxin, Apoptosis, Cytotoxin, Cytostatic

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**
 - Aim 1: Develop BB2 agonist (GRP) and GnRH-R agonist (Lys6-GnRH) conjugates with the endosome disrupter, listeriolysin O (LLO); the ribosome inactivating protein, Gelonin (cytotoxic); and doxorubicin, a cytostatic toxin that preferentially targets mitotic cells
 - 1) Initial Synthesis Completion (Vagner and Renquist)
 - Initial Target Completion: Year 2 Quarter 1
 - Percent Complete: 100%
 - 2) Expected Additional Optimization Target (Selvaraj and Renquist)
 - Initial Target Completion: Year 3 Quarter 4
 - Percent Complete: 100%
 - 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. We expected this aim to continue throughout the 3-year project duration, with milestones reached in Years 1, 2, and 3.
 - 1) Experiment 1: In Vitro Test of Linker Target Completion (Selvaraj and Renquist)
 - Initial Target Completion: Year 3 Quarter 4

- Percent Complete: 100%
 - 2) Experiment 2: Identify the maximum ineffective concentration of ligand directed endosome disruptor. (Selvaraj and Renquist)
 - Initial Target Completion: Year 2 Quarter 1
 - Percent Complete: 100%
 - 3) Experiment 3: Minimum effective concentration of ligand directed toxins in presence and absence of ligand directed endosome disruptors. (Selvaraj and Renquist)
 - Initial Target Completion: Year 2 Quarter 3
 - Percent Complete: 100%
 - 4) Experiment 4: Identify the minimum concentration of ligand directed LLO that maximally encourages ligand directed toxin efficacy. (Selvaraj and Renquist)
 - Initial Target Completion: Year 2 Quarter 3
 - Percent Complete 100%
- Aim 3: Perform *in vivo* testing of two combined targeted toxin/targeted endosome disruptor treatment in mice bearing xenografts of the MDA-PCa-2b and PC-3 prostate cancer cell line.
 - 1) Experiment 1: Test the *in vivo* efficacy of targeted endosome disruptors and targeted toxins. (Renquist)
 - Initial Target Completion: Year 2 Quarter 4
 - Percent Complete: 25%
 - The difficulty in creating a GnRH-LLO conjugate that maintained lytic capacity caused this project to stall.
 - 2) Experiment 2: Optimize a treatment paradigm to eliminate prostate cancer tumor burden. (Renquist)
 - Initial Target Completion: Year 3 Quarter 4
 - Percent Complete: 0%
- **What was accomplished under these goals?**
 - Aim 1: Develop BB2 agonist (GRP) and GnRH-R agonist (Lys6-GnRH) conjugates with the endosome disrupter, listeriolysin O (LLO); the ribosome inactivating protein, Saporin (cytotoxic); and doxorubicin, a cytostatic toxin that preferentially targets mitotic cells

Major Activities, Accomplishments, and Major Findings:

 - 1) GnRH-LLO, GRP-LLO, GnRH-Saporin, and GRP-Saporin have been synthesized. (Vagner)
 - Accomplishment: Successful synthesis of 4 conjugates

- 2) GnRH-LLO and GRP-LLO *in vitro* efficacy was established in red blood cell lysate assay. (Renquist)
- Accomplishment: Successful *in vitro* testing of LLO conjugates.
 - Major Finding: Conjugating LLO to GnRH and GRP decreased EC50 for lytic potential about 10-fold. This minor affect on lytic potential should not limit efficacy when internalized into vesicles.

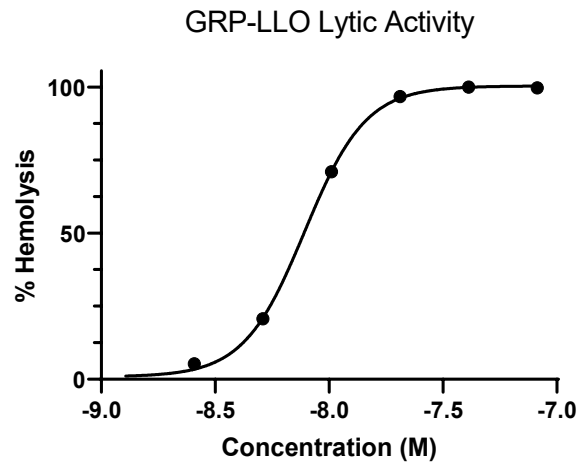


Figure 1. GRP-LLO is hemolytic at concentrations greater than 10 nM.

- Other achievements: Developed an FPLC method to purify 0.3-0.5 mg of LLO/batch.
- 3) GnRH-Saporin and GRP-Saporin *in vitro* efficacy was established by rabbit reticulocyte lysate protein synthesis assay. (Renquist)
- Accomplishment: Successful *in vitro* testing of Saporin conjugates

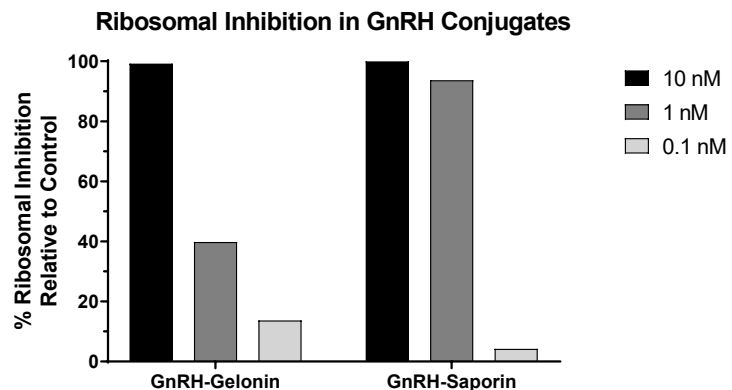


Figure 2. GnRH-Saporin conjugates were more effective at inhibiting protein synthesis than GnRH-Gelonin conjugates. In turn all future studies use GnRH-Saporin conjugates.

- Major Finding: Neither conjugation with GnRH nor GRP affected the inhibition of *in vitro* protein synthesis by Saporin.

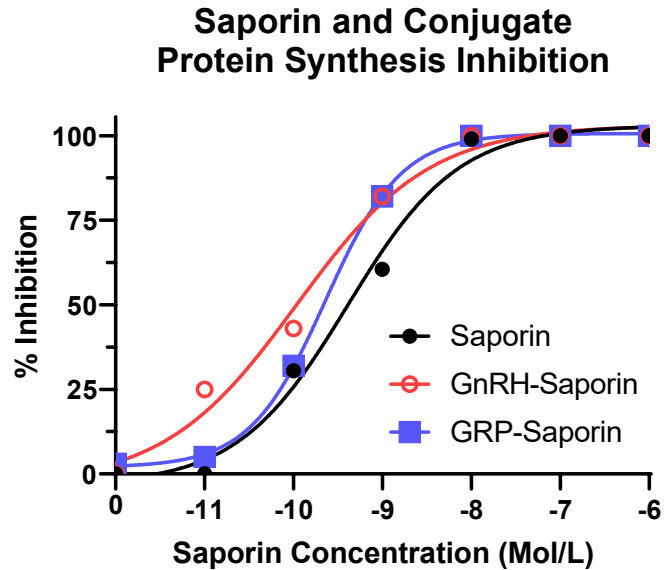


Figure 3. Neither conjugation to GRP nor GnRH affected the ability of Saporin to inhibit protein synthesis.

Stated goal not yet met: We are currently synthesizing the GnRH-doxorubicin and GRP-doxorubicin conjugates. These conjugates were not involved in the completion of Aim 2 and only necessary for Aim 3.

- 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. We expected this aim to continue throughout the 3-year project duration, with milestones reached in Years 1, 2, and 3.

Major Activities, Accomplishments, and Major Findings:

- Experiment 1: In Vitro Test of Linker Target Completion (Selvaraj and Renquist)
 - Accomplishment: Successful synthesis of Listeriolysin O and Saporin ligand conjugates with minimal loss of Listeriolysin O lytic activity or Saporin protein synthesis inhibition.

- 2) Experiment 2: Identify the maximum ineffective concentration of ligand directed endosome disruptor. (Selvaraj and Renquist)
- Major Findings: The maximum ineffective dose of GnRH-LLO is 10 nM in two prostate cancer cell lines (PC3 cells and DU145 cells).

Toxicity of GnRH-LLO in DU145 Cells

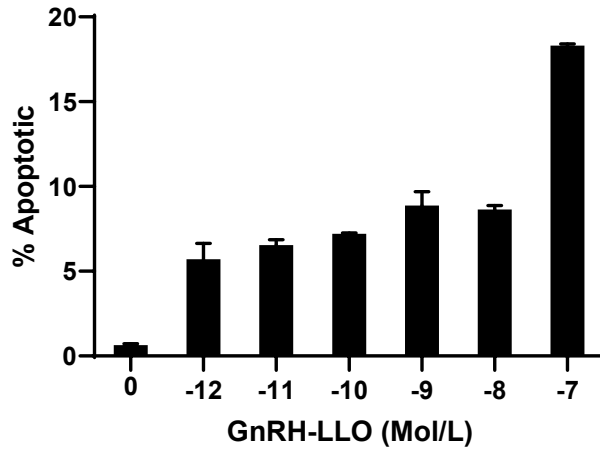


Figure 4. GnRH-LLO induces mild apoptosis in DU145 cells up to a concentration of 1 nM, above that apoptosis increases.

Toxicity of GnRH-LLO in PC3 Cells

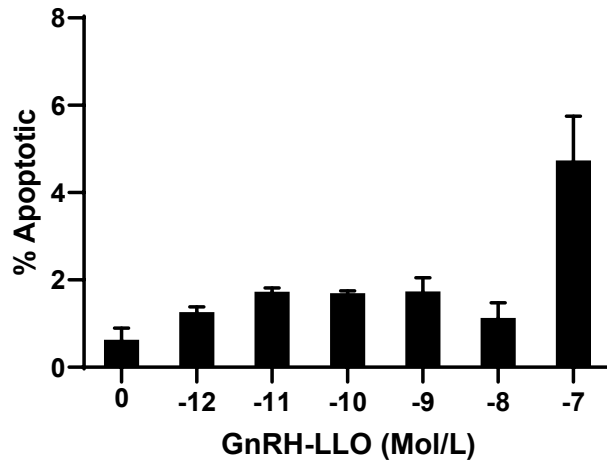


Figure 5. GnRH-LLO induces mild apoptosis in PC3 cells up to a concentration of 10 nM, above that apoptosis increases.

3) Experiment 3: Minimum effective concentration of ligand directed toxins in presence and absence of ligand directed endosome disruptors. (Selvaraj and Renquist)

- DU145:

- Major Findings: GnRH-LLO (10 nM) does not improve the efficacy of GRP-Saporin in DU145 cells.

GnRH-LLO does not improve efficacy of GRP-Saporin in DU145 Cells

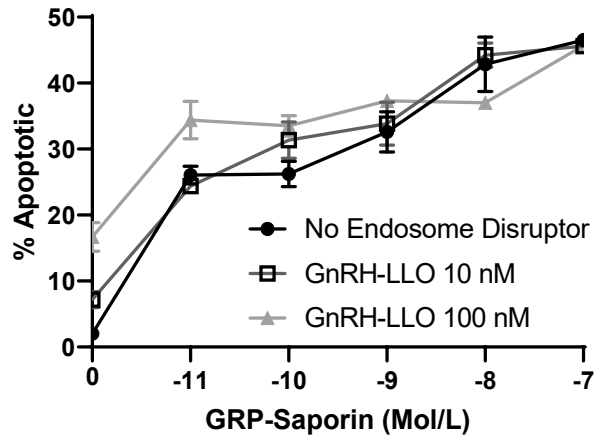


Figure 6. GnRH-LLO did not significantly increase the efficacy of GRP-Saporin in DU145 cells.

- PC3:

- Major Findings: GnRH-LLO (10 nM) decreases the effective dose of GRP-Saporin from 10 pM to 1 fM (10,000 X)

GnRH-LLO Enhances Efficacy of GRP-Saporin in PC3 Cells

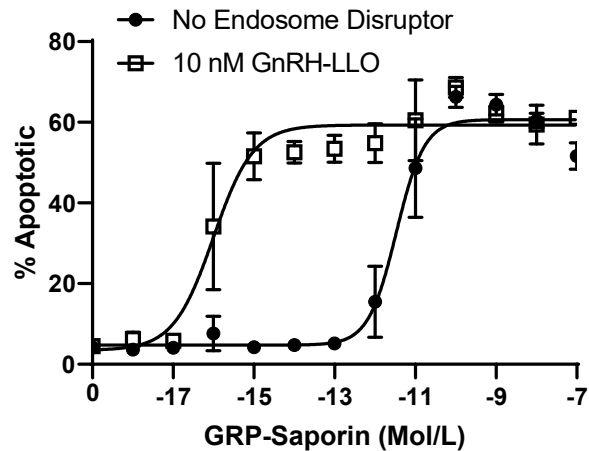


Figure 7. GnRH-LLO significantly increased the efficacy of GRP-Saporin in PC3 cells.

- 4) Experiment 4: Identify the minimum concentration of ligand directed LLO that maximally encourages ligand directed toxin efficacy. (Selvaraj and Renquist)
- DU145:
 - Major Findings: There is no difference in improvement of efficacy of GRP-Saporin between 10 and 100 nM GnRH-LLO. GRP-LLO did not improve the efficacy of GnRH Saporin.
 - PC3:
 - Major Findings: There is no difference in improvement of efficacy of GRP-Saporin between 10 and 100 nM GnRH-LLO. GRP-LLO did not improve the efficacy of GnRH Saporin.
- Aim 3: Perform *in vivo* testing of two combined targeted toxin/targeted endosome disruptor treatment in mice bearing xenografts of the MDA-PCa-2b and PC-3 prostate cancer cell line.
- 1) Experiment 1: Test the *in vivo* efficacy of targeted endosome disruptors and targeted toxins. (Renquist)
- Major Activities: Growing donor tissues in mice, transferred donor tissue to recipient mice, treated recipient mice with a single dose of GRP-Saporin GnRH-LLO. At a low dose the tumor continued to advance, at the higher dose, the tumor stopped growing and did not advanced (Figure 8).

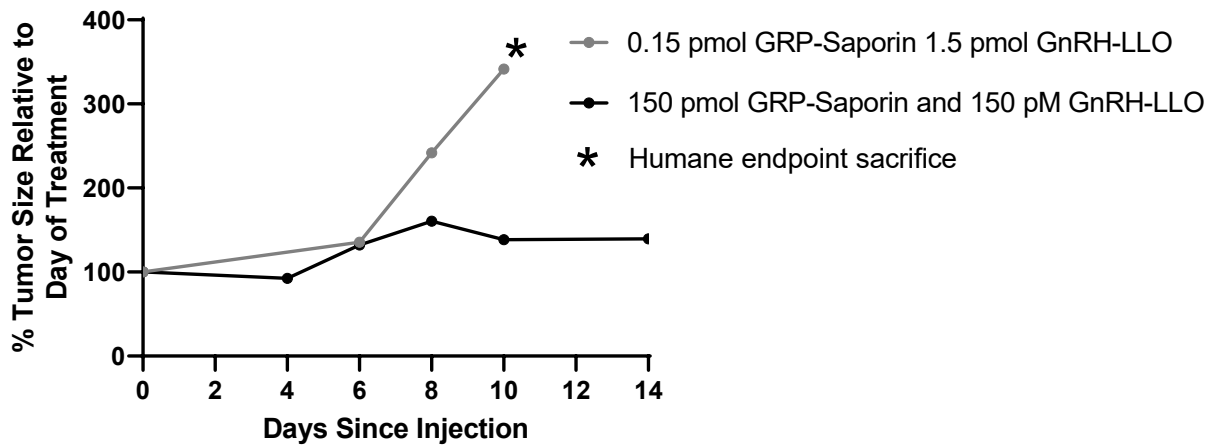


Figure 8. High dose conjugate cocktail limits tumor progression in a mouse model of human prostate cancer.

- Major Activities: Upon a successful first test, we needed significant quantities of LLO. We grew, induced, and isolated 3.2 mg of LLO from 100 L of E.Coli.
 - We first lyse the E.Coli (lysate is black). We then precipitate using Ammonium Sulfate (0-30% and

30-50% ammonium sulfate had most of the activity). We next put the precipitate through a his-trap column (FPLC). The flow through (blue) has little activity, but the SEC Load (aquamarine), which is the his-trap collection has improved activity relative to what we loaded onto the his-trap column (purple). Finally, we loaded this onto a size exclusion column, which allowed use to improve activity (measured as red blood cell hemolysis by nearly 4 times) (Figure 9).

072320 LLO-HIS RBC Lysis Assay Relative to Max

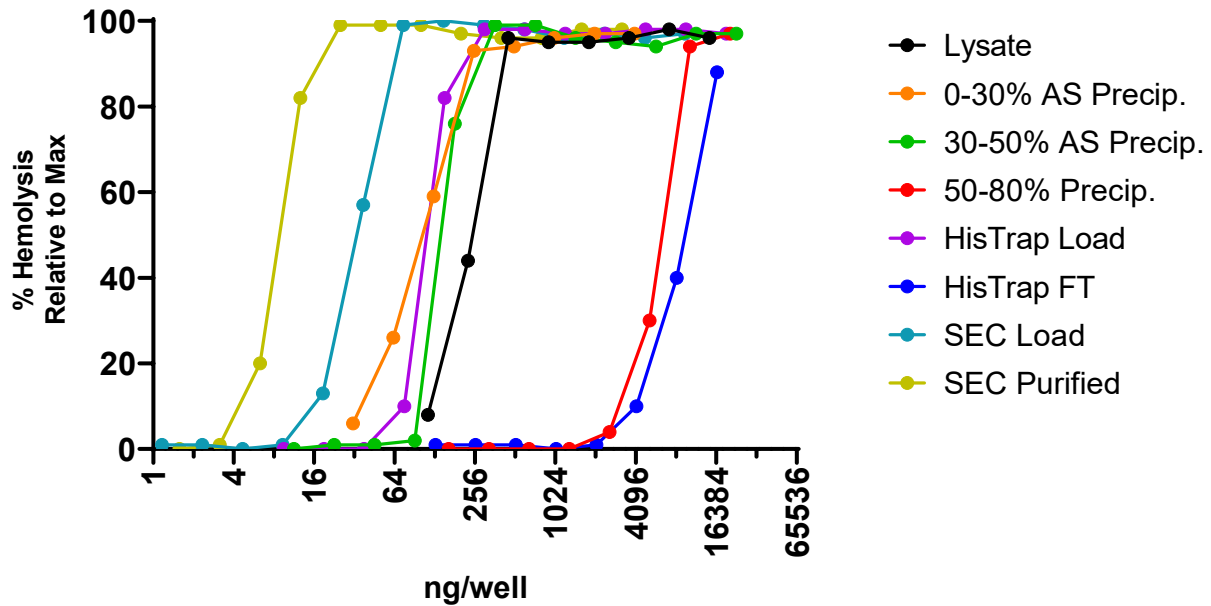


Figure 9. Purification steps and improvements in lytic activity of Listeriolysin O.

- Experiment 2: Optimize a treatment paradigm to eliminate prostate cancer tumor burden. (Renquist)

Stated goal not yet met: We have conducted preliminary tests in mice to validate the model and potential therapeutic potential. These studies were very promising.

We have created 3.2 mg of LLO for conjugation with GnRH. To purchase this commercially would have cost \$32,000.

We were unable to re-make GnRH-LLO that maintained lytic potential despite 4 attempts. Future research should focus on identifying potent membrane disruptors that can be targeted to cell.

- **What opportunities for training and professional development has the project provided?**
 - Renquist Laboratory: In the Renquist lab Kyle Kentch and Susma Ghimire have undergone significant personal training. Kyle Kentch has learned how to transfect E.Coli that express plasmids that allow for inducible expression of proteins, grow E. Coli, and apply FPLC to purify proteins from these E. Coli. Susma Ghimire has grown PC3 cells and transplanted them into nude donor mice. Kyle Kentch and 3 undergraduate students have helped to monitor tumor growth.
 - Vagner Laboratory: In the Vagner lab, Renata Patek and Zheny Zhang have developed chemistry for conjugation of hydrophilic polyethylene linkers and azine-alkyne conjugation as orthogonal scheme to disulfide linkage. Both have also undergone annual training by NMR Core center to use automatic 400MHz and 500MHz NMR.
 - Selvaraj Laboratory: In the Selvaraj lab, two MS students Bailey Lester, Keila Acevedo were trained by Dr. Selvaraj to grow and propagate cancer cell lines, analyze apoptotic cell percentages using cytometry application. A PhD student, Gabriel Akerele was trained in prostate cancer research methodology and apoptosis assay. The PhD student, Gabriel Akerele, was later hired as a postdoc
- **How were the results disseminated to communities of interest?**
 - Dr. Renquist has given a seminar at the University of Arizona Cancer center on this work to enhance targeted toxins by promoting endosomal escape. A manuscript is in preparation to describe conjugate synthesis and *in vitro* findings. This manuscript is a combination of the manuscripts proposed for Aims 1 and 2 and will be placed into BioRxiv immediately upon completion.
 - Dr. Selvaraj: A poster was presented at the College of Agriculture and Environmental Sciences, University of Georgia undergraduate research forum. A high school student presented a poster at the Summer research internship forum at University of Georgia.
 - Dr. Vagner: A poster was presented at the University of Arizona, the Bio5 Institute and UA Cancer Center
- **What do you plan to do during the next reporting period to accomplish the goals?**
 - N/A

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
 - We showed that targeted toxin efficacy can be greatly improved with targeted endosome disruptors.

- **What was the impact on other disciplines?**
 - There are other cases for which targeted ablation of cells may be beneficial. (i.e. benign hyperplasia, hyperthyroidism, Cushing's syndrome). Validation of the simultaneous application of a targeted endosome disruptor and targeted toxin could be applied to these human conditions.

Our findings could also be applied to the ablation of cells in animals. A primary focus would be the development on an injectable sterilant that could be applied to companion animals, to non-lethally control wild populations, or to improve production efficiency in animal agriculture.
- **What was the impact on technology transfer?**
 - We have submitted an invention disclosure that is bolstered by our findings from this study. That invention disclosure specifically addresses the simultaneous delivery of a targeted endosome disruptor to improve the efficacy of a targeted toxin. We anticipate that this technology can be applied across all cancers.
- **What was the impact on society beyond science and technology?**
 - Application of this knowledge to animal populations would limit companion animal overpopulation and the euthanasia of millions of companion animals yearly and allow for non-lethal control of wild animal populations. Application to control animal populations could be applied to limit environmental damage caused by overgrazing in national parks and wildlife areas.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**
 - Nothing to Report.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
 - We were delayed in LLO conjugate synthesis due to difficult in LLO purification from E.Coli in significant quantities. Importantly, we developed techniques that allowed us to purify large 3-4 mg batches which would be valued at \$3000-\$5000/batch.
 - Creating GnRH-LLO conjugates that maintained lytic potential was problematic.
- **Changes that had a significant impact on expenditures**
 - Nothing to Report
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
 - Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**
 - **Journal publications.**
 - 1) Miller, K.E., K.K. Kentch, R. Patek, Z. Zhang, R. Selvaraj, J. Vagner, R. Selvaraj, and B.J. Renquist. 2021. Improved efficacy of targeted ribosome inactivating proteins with simultaneous targeting of endosome disruptors. In Preparation.
 - **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**

Quick and Rapid method for purification of large quantities of LLO.
- **Inventions, patent applications, and/or licenses**

Nothing to Report
- **Other Products**

E.Coli that can be induced to produce LLO and Saporin

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?

Name:	<i>Benjamin Renquist</i>
Project Role:	<i>Principal Investigator</i>
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 Saporin and the tests of LLO and Saporin Efficacy. This involved a good deal of problem solving and evaluating the literature. I have further guided all <i>in vivo</i> studies of efficacy.
Funding Support:	University of Arizona

Name:	<i>Kyle Kentch</i>
Project Role:	<i>Research Staff</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Kyle led the purification of Listeriolysin O and Saporin and conducted tests of LLO and Saporin conjugate <i>in vitro</i> efficacy. He has also led the development of tumors in mice and initial studies of <i>in vivo</i> efficacy.
Funding Support:	Found Animals Foundation. They funded research that is using Gelonin and LLO conjugates for the development of an injectable sterilant.

Name:	<i>Susma Ghimire</i>
Project Role:	<i>Research Staff</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Susma has initiated our in vivo work with nude mice and begun to grow tumors in donor mice for transplant into study mice.
Funding Support:	Arizona Biomedical Research Center

Name:	Ramesh Selvaraj
Project Role:	<i>Principal Investigator</i>
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:	<i>Undergraduate Student researcher</i>
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:	<i>Graduate Student researcher</i>
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:	<i>Graduate Student researcher</i>
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:	Gabriel Akerele
Project Role:	<i>Graduate Student researcher</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Gabriel was trained in apoptosis assay and prostate cell culture
Funding Support:	

Name:	Kamel Zaghoul
Project Role:	<i>Graduate Student researcher</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6

Contribution to Project:	Kamel 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:	<i>Principal Investigator</i>
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 LLO, Saporin, and Gelonin conjugates with GnRH, BB2 and other targeting ligands, upscaled solid-phase synthesis of GnRH, BB2, and other targeting ligands. I have developed conjugation chemistries based on sterically hindered disulphide and thioether linkers.
Funding Support:	

Name:	Zhenyu Zhang
Project Role:	<i>Research Specialist</i>
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	Zhenyu performed the synthesis of LLO, Saporin, and Gelonin conjugates, solid-phase synthesis of targeting ligands (GNRH, BB2 and other), conjugates and intermediates purification and analysis
Funding Support:	

- **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:* Dr. Renquist has a new grant to better understand diet effects on lactation in heat stressed mice (Phibro).
 - Selvaraj: Nothing to Report
 - Vagner: Nothing to Report
- **What other organizations were involved as partners?**
 - 1) **Organization Name:** University of Arizona
 - 2) **Location of Organization:** Tucson, AZ
 - 3) **Partner's contribution to the project** (*identify one or more*)
 - **In-kind support:** Provided the FPLC necessary to purify proteins to the Renquist Laboratory.