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14. ABSTRACT
   The objective of this project is to develop a novel RNA interference-based therapy for fatty liver diseases and steatohepatitis. Our strategy is to develop a means of targeting siRNA molecules to relevant cells of the liver in order to silence genes that play key roles in inflammatory, metabolic or other pathways that cause disease initiation or progression. The key specific goal of our project is to develop a simple 2 component delivery vehicle for such gene silencing in liver. During this year we have found that covalent coupling of a moiety (e.g., cholesterol) that promotes entry into cells directly onto the siRNA (forming "self delivery "sd" RNAs) rather than directly onto glucan shells that target Kupffer cells is a superb approach. Our priority formulations which we will continue to advance in this project are therefore sdRNAs that can be used alone or encapsulated by glucan shells to selectively target Kupffer cells, as we originally proposed. The data we produced this fourth quarter of year 1 show nearly 100% efficiency of cholesterol-siRNA entering hepatic cells in mice following injection by the subcutaneous route at a dose of 10mg/kg. The cholesterol conjugate can be further modified with bioconjugates designed to confer specific cell type delivery and silencing. Thus, by injecting sdRNA alone versus sdRNA encapsulated within glucan shells (GeRPs) we will be able to confer hepatocyte vs Kupffer cell-specific delivery for target gene silencing and alleviating steatohepatitis.

15. SUBJECT TERMS
   Fatty liver disease, RNA interference, Kupffer cells, hepatocytes, siRNA delivery

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>4</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>4</td>
</tr>
<tr>
<td>3. Accomplishments</td>
<td>5</td>
</tr>
<tr>
<td>4. Impact</td>
<td>18</td>
</tr>
<tr>
<td>5. Changes/Problems</td>
<td>18</td>
</tr>
<tr>
<td>6. Products</td>
<td>19</td>
</tr>
<tr>
<td>7. Participants &amp; Other Collaborating Organizations</td>
<td>19</td>
</tr>
<tr>
<td>8. Special Reporting Requirements</td>
<td>20</td>
</tr>
<tr>
<td>9. Appendices</td>
<td>20</td>
</tr>
</tbody>
</table>
1. INTRODUCTION:

Based on a dire need for novel therapies for a significant and burgeoning disease, the overall objective of this project continues to be development of a novel RNA interference-based treatment for fatty and inflamed liver diseases such as NASH and alcoholic liver disease. Our strategy is to develop a means of targeting siRNA molecules to relevant cells of the liver in order to silence genes that play key roles in inflammatory, metabolic or other cellular pathways that contribute to disease initiation or progression. We had initially developed a three component glucan-shell based nanoparticle that could deliver siRNA specifically to Kupffer cells, resident macrophages of the liver. Our goal in this project continues to be to simplify this 3 component vehicle which would be difficult to manufacture into a 2 component siRNA delivery vehicle. Our initial approach has been to covalently attach moieties that allow entry into cells (“entry molecule” such as weak bases or hydrophobic molecules) on the glucan shells that encapsulate the siRNA. However, studies during this period revealed optimal siRNA binding of these particles at low pH rather than under physiological serum conditions and therefore less suitable for intravenous delivery. We therefore shifted the covalent attachment of the “entry molecule” such as cholesterol to the siRNA rather than the glucan shell, creating new siRNA formulations (self-delivering, or sdRNAs). These sdRNAs can be further modified with bioconjugates designed to confer specific cell type delivery and silencing. Studies we have performed over the 4th quarter of year 1 of this project using a cholesterol conjugated sdRNA demonstrated virtually 100% efficient delivery and bio-distribution to multiple liver cell types at a concentration of 10mg/kg in mice. An additional advantage of these formulations is the possibility of delivery by subcutaneous injection, thus facilitating potential clinical application. Future studies will employ bioconjugates including n-acetyl galactosamine (GalNac) conjugates that should confer hepatocyte specificity or others tailored for Kupffer cell-specific delivery. In parallel we will encapsulate sdRNAs within glucan shells, which are readily phagocytosed by Kupffer cells and macrophages as demonstrated in our earlier studies, for targeted delivery of the sdRNA to these phagocytes in liver as originally proposed. We are in position to move to testing gene silencing in vivo by these new priority formulations during the next project period.

2. KEYWORDS:

Fatty liver disease, RNA interference, Kupffer cells, hepatocytes, siRNA delivery, glucan particles (GP)
3. ACCOMPLISHMENTS:

**Major goals and objectives:**

**Specific Aim 1:** Design, synthesize and functionally screen novel glucan shell formulations (GeRPs) for siRNA delivery.

  Major Task 1: Develop lead candidates for in vivo functional studies in Aim 2
  Target date 9/30/16 Completed 6/30/16

**Specific Aim 2:** Test candidate GeRP formulations in diet-induced and alcoholic fatty liver disease models.

  Major Task 3: In vivo studies of GeRP-mediated NFkB on NASH and alcoholic steatohepatitis models.
  Target date 9/30/16 has been delayed to the next quarter due to required testing of bio-distribution of priority formulations prior to gene silencing work—see section 5.

**Accomplishments under these goals:**

*Design and synthesis of initial Endoporter-based peptide-modified GeRP formulations:*

Our initial approach toward developing a novel siRNA delivery vehicle was to design a glucan shell-based particle modified with covalently attached moieties that could supplant need for Endoporter in our previous formulation. Thus we prepared a library of small molecule amines as well as short peptide sequences (Table 1) that we expected might confer suitable siRNA binding and release capacity upon conjugation to glucan shell while preserving targeting specificity to phagocytic cells observed with our existing GeRP formulations.

<table>
<thead>
<tr>
<th>Small Molecule Amines&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Selected Peptides&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylenediamine (E)</td>
<td>LHLLHHLLHHLHHL (EP14)</td>
</tr>
<tr>
<td>Histamine (H)</td>
<td>LHHLL (EP5)</td>
</tr>
<tr>
<td>E:H (75:25)</td>
<td>His15</td>
</tr>
<tr>
<td>E:H (50:50)</td>
<td>MethylHis15</td>
</tr>
<tr>
<td>E:H (25:75)</td>
<td>His7</td>
</tr>
<tr>
<td>E:Leu5 (50:50)</td>
<td>His15:Leu5 (50:50)</td>
</tr>
<tr>
<td>E:Leu15 (50:50)</td>
<td>His15:Leu15 (50:50)</td>
</tr>
</tbody>
</table>

Table 1: Small-molecule amines and sequences of peptides used in the present studies. Abbreviations are presented in parentheses. For particles modified with two different amines and/or peptides, the ratio given in parentheses is the theoretical molar ratio of the two compounds.

These were coupled to beta-glucan shells via reductive amination chemistry as described in our proposal. Efficiency of coupling to glucan particles (GPs) was assessed by fluorescamine assay
for amines, or bicinchoninic acid assay (BCA) for peptides. To ensure that the conjugation did not cause significant changes in physical properties of the particles (such as aggregation that might adversely affect cell uptake) modified GP preparations were examined for particle size by confocal microscopy, and Zeta potentials of peptide-modified GPs were determined with a Malvern Zetasizer Nano-ZS instrument. Based on these assessments covalent modification of GPs did not change these properties.

To determine the ability of the amine- and peptide-modified GPs to electrostatically interact with siRNA, we incubated fluorescently-labeled siRNA with the modified GPs at different concentrations (Figure 1A). Alternatively, modified GPs were loaded with different concentrations of fluorescently-labeled siRNA (Figures 1B-D). As predicted, the different amine- and peptide-modified particles bind siRNA with varying degrees of strength (Figure 1). Unmodified GPs did not bind siRNA at any of the concentrations tested (Figure 1B).

**Figure 1: In Vitro siRNA binding assays of covalent GP formulations.** Particles were loaded with fluorescently labeled siRNA (Dy547-siRNA, Dharmacon, Pittsburgh, PA) by incubating in sodium acetate buffer (30 mM, pH 4.8) for 20 min at room temperature. Particles loaded with siRNA were sedimented by centrifugation at 9000 rpm for 5 min and the supernatant was assessed for siRNA content by measuring the fluorescence on a microplate reader. % siRNA binding as well as % free siRNA was calculated relative to the siRNA control that did not contain particles (siRNA alone).
These results demonstrate the feasibility of synthesizing covalently modified glucan particles with peptide and amine moieties that confer siRNA binding capability.

**Toxicity screening of novel formulations:**

Our original glucan particle (GP) formulation is well tolerated by cells and animals even at high doses (Tesz, et al., Biochem J 436:551). To rule out toxic effects arising in novel covalently modified formulations, J774A (cultured macrophage cell line) cells or peritoneal exudate cells (PECs, primary macrophages from thyoglycollate treated mouse peritoneum) were treated with novel GP formulations at various doses and effects on cell viability assessed by MTT assay. Figure 2 shows no significant increases in toxicity compared to unmodified GP.

**Figure 2:** Modified GPs are non-toxic *in vitro*. Viability of (A) J774A.1 cells and (B) PECs treated with various concentrations of modified GPs for 48 h was measured using MTT cell proliferation assay. Results are mean ± s.e.m.
**Novel formulations are taken up by macrophages:**

In order to verify that modified GP formulations are efficiently taken up by phagocytosis into macrophages, novel GP preparations were labeled with FITC for microscopic visualization. After 48 hour incubation with FITC-GPs PECs were stained with fluorescent F4/80 antibody to identify macrophages. (Figure 2). Results show that a majority of F4/80 positive macrophages contain multiple FITC-GPs for all formulations tested. These results indicate that surface modification of GPs does not inhibit recognition and uptake by macrophages.

**Figure 3: Modified GPs are phagocytosed by macrophages in vitro.** PECs were treated with FITC-labeled modified GPs loaded with siRNA in vitro for 48 h. Confocal microscopy shows modified GPs (green) present in F4/80-positive macrophages (red). Nuclei were stained with DAPI (blue) (60x magnification, scale bar: 20 µm).

**In vivo silencing with the novel chemically modified GP formulations:**

As an initial test of efficacy, silencing of a macrophage target gene in vivo was assessed following intraperitoneal injection of standard and novel GP formulations loaded with scrambled control siRNA or a validated siRNA targeting F4/80. RNA expression analysis of PECs isolated
after 6 daily injections showed efficient silencing by the previous GP (GeRP) formulation (Figure 4). Novel formulations GP-EP14 and GP-His15 also showed significant efficacy.

Figure 4: Modified GPs can silence genes in macrophages in vivo in healthy, lean mice. (A) Time line of modified GP administration and isolation of PECs. Briefly, 8-week old C57BL6/J mice were injected once a day for 5 days with modified GPs or GeRPs loaded with either Scr (black) or F4/80 (grey) siRNA. On day 6, mice were sacrificed and PECs were isolated. Total RNA was extracted and F4/80 expression was measured by real-time PCR in PECs. mRNA expression data from PECs isolated from mice treated with (B) GP-His15, (C) GP-His7, (D) GP-Histamine, (E) GP-Ethylene diamine, (F) GP-EP14, and (G) GeRPs. n = 5-9, statistical significance was determined by student’s t-test. *p < 0.05, ***p < 0.001. Results are mean ± s.e.m.

RNA binding properties of novel chemically modified GP formulations:

Prior to proceeding with a lead candidate GP formulation in vivo using intravenous delivery to access liver Kupffer cells, it is necessary to carefully assess the siRNA binding kinetics under physiological pH and salt conditions. Optimal function of these GPs in vivo would depend on a number of factors, including stable association of siRNA cargo as the particles transit through the bloodstream and accumulate in the liver. In this progress period, we thus assessed siRNA binding and release characteristics under a number of conditions including approximations of serum pH and ionic strength conditions.
We first assessed siRNA binding properties of a panel of amine-modified GP formulations under physiological and low pH conditions. Time dependence of binding, concentration dependence of binding, pH dependence of binding and salt concentration dependence were all studied independently and in combinations and are summarized in representative data sets below (Figure 5).

![Graphs showing binding of siRNA to amine-conjugated GPs under different conditions](image)

**Figure 5: Binding of siRNA to amine-conjugated GPs requires low pH.** Indicated concentrations of fluorescent-labeled siRNA was incubated with the modified GP formulations (1 mg/ml) in the indicated buffers and free siRNA assessed by quantitation of fluorescence remaining in the supernatant. PBS, phosphate-buffered saline, pH 7.4, 120 mM NaCl; NaAc, 30 mM sodium acetate, pH 4.8

These comprehensive results confirm a requirement for low pH to form stable GP-siRNA complexes with these amine-modified GP formulations, with suboptimal binding at physiological pH. We next assessed the stability of such complexes, formed at low pH, upon dilution into buffers at physiological pH (Figure 6).
Figure 6: GP-siRNA complexes dissociate upon dilution into physiological pH. Modified GP-siRNA complexes were formed in NaAc buffer, pH 4.8, and subsequently diluted into solutions with the indicated ratios of NaAc and PBS (pH 7.4, panel A), or acidified water (pH 3.0, panel B). Free siRNA was quantitated by measurement of fluorescence in the supernatant at the indicated times.

These results indicate essentially quantitative release in less than 30 minutes of exposure to increased pH. To assess the likelihood of stable siRNA binding under physiological serum conditions we also assessed the binding of fluorescent siRNA to amine- or peptide-modified GP
formulations including lead candidates GP-His15 and GP-EP 14 under varying conditions of pH and ionic strength (Figure 7). These results revealed similar pH-sensitive properties of amine-modified and peptide-modified GP formulations. We further assessed release kinetics of siRNA from novel GP formulations under various pH conditions (Figure 8).

Figure 7: Lack of stable siRNA association with candidate GP formulations in physiological conditions. Fluorescent-labeled siRNA was incubated with the indicated concentrations of modified GP formulations and binding assessed by quantitation of fluorescence remaining in the supernatant. HBSS, Hepes-buffered saline solution, pH 7.6, 120 mM NaCl; NaAc, 30 mM sodium acetate, pH 4.8
Collectively, these results suggest that stable, long term binding of siRNA to these weak base GP formulations is optimal under conditions of low pH or very low ionic strength (approximately 10% of physiological saline), and are unlikely to mediate optimal siRNA delivery upon exposure to serum after intravenous injection.

Figure 8: Rapid release of siRNA from GPs at physiological pH. Fluorescent-labeled siRNA-GP complexes were diluted into PBS (pH 7.4), NaAc (pH 4.8) or the indicated ratios of each. siRNA release was assessed by quantitation of fluorescence remaining in the supernatant. Samples were taken from supernatants at the indicated times.
Based on these data, we hypothesized that optimal siRNA delivery would be achieved by attaching the moiety that mediates cell membrane translocation (e.g., the weak bases in the formulations above) directly to the siRNA rather than to the GP, and then either using such modified siRNA directly or encapsulating it within the GPs. Previous experiments with such formulations indicate high success in achieving delivery at physiological pH and physiological salt conditions, but this had never been tried in the context of GP encapsulation.

We therefore directed our efforts towards use of such modified siRNA formulations. These formulations, referred to as self-delivering siRNAs (sdRNAs) combine incorporation of modified ribonucleotide bases in the siRNA sequence with capability for attaching targeting moieties at the 3' terminus of the sense strand of the RNA. We initiated work in the 4th quarter of the project using the Cholesterol-modified siRNA shown at the top in Figure 9, and two other possible formulations are also shown in Figure 9.

**Figure 9: Structure of conjugated self-delivering siRNA (sdRNA)**
We have initiated studies of biodistribution of the Chol-sdRNA formulation which was found to accumulate in liver following subcutaneous injection (area of abdomen, rectangle in Figure 9) in preliminary studies, and have successfully demonstrated delivery of the Cy3-labeled sdRNA to cell types in liver including hepatocytes and Kupffer cells. The experiment was carried out as illustrated in Figure 10, and results are shown in Figure 11 below.

- Animal strain/age: 12 weeks old male FVB/N mice
- siRNA type: Cholesterol modified sdRNA with Cy3 label.
- Dosage: 10mg/kg, 1mg/kg, PBS(Control)
- Injection: Single subcutaneous injection from belly
- End point: After 48hrs from injections.
- Sacrifice and Tissue processing:
  - Liver perfusion and digestion for isolating Kupffer cells and hepatocytes
  - Harvesting SubQ fat depot near and far from the site of injection, following with isolation of primary adipocytes and SVF fraction.
  - Harvesting the site of injection (belly skin) for histology.

**Figure 10: Experimental design for sdRNA injection experiment.**

Results of this experiment were extremely successful and highly encouraging. The data below shows extensive and dose dependent labeling of hepatocytes by the Cy3-labeled cholesterol-sdRNA that was injected subcutaneously (Figure 11, upper panels). Extensive uptake of Cy3-sdRNA could also be detected in F4/80-positive Kupffer cells at the 10mg/kg dose.

These data demonstrate that conjugating the siRNA itself with an “entry molecule”, producing an sdRNA molecule, targets liver effectively from a subcutaneous injection. Moreover, encapsulating such a sdRNA molecule with glucan shells would therefore provide a 2 component siRNA delivery vehicle that could be administered in vivo (in that case, by i.v. injection) to selectively target Kupffer cells in the liver, which is the original goal of this overall project. We therefore have identified this 2 component formulation as our priority formulation moving forward. The additional benefit from this strategy is that the sdRNA alone, in non encapsulated form, itself is a powerful 1 component vehicle for gene silencing in liver, albeit not cell specific for Kupffer cells as is the encapsulated form. Taken together, these exciting findings provide us with powerful formulations to proceed with gene silencing in vivo for alleviation of NASH and liver inflammation and fibrosis.
To further characterize and quantify non-hepatocyte liver cells that take up chol-sdRNA, we performed FACS analyses of non-parenchymal (non-hepatocyte) cell fractions from sdRNA injected animals (Figure 12) using markers for Kupffer cells, endothelial cells and stellate cells. The results show that more than 40% of CD31-positive fraction of non-parenchymal cells (consisting of endothelial and Kupffer cells) are labeled with Cy3-sdRNA that was subcutaneously injected, as is approximately 20% of the Kupffer cell (F4/80+) fraction (Panels A,B). To determine distribution in the total cell population we subtracted the double positive (F4/80 and CD31 positive cells) from the whole CD31 positive population and plot the percentage Cy3+ cells for each Panel C). These results show that of the total non-parenchymal cell population, approximately 12% of cells are Cy3-sdRNA positive under these conditions, and
these consist of approximately equal proportions of Kupffer and endothelial cells. We could not detect any uptake of sdRNA by stellate cells under these conditions (data not shown). These data are for a 1mg/kg dose of Cy3-sdRNA which we know from the above experiment is much less effective than 10mg/kg, and results from this higher dose are currently being assessed. We expect virtually full 100% efficiency of targeting liver cells at this higher dose.

These results demonstrate that cholesterol-conjugated sdRNA is taken up by several cell types in liver including hepatocytes, endothelial cells and Kupffer cells.

Figure 12: FACS analysis of non-parenchymal liver cell uptake of cholesterol-sdRNA. Mice received a single subcutaneous injection of 1 mg/kg cholesterol-sdRNA (n=3) or PBS (n=2). After 48 hours non-parenchymal fractions from perfused livers were isolated and stained for FACS using labeled antibodies to CD31 (endothelial and Kupffer cell marker) and F4/80 (Kupffer cell marker). Panel A depicts individual sample scatter plots for CD31+ (endothelial + Kupffer cells,) and F4/80+ (Kupffer cell) fractions, and histograms showing fractions of Cy3-sdRNA+ cells in marker-positive populations. Panel B shows percent Cy3+ cells in CD31+ fraction (blue bars) and Kupffer cells (orange bars). Panel C shows percent Cy3+ (sdRNA) labeled cells of total non-parenchymal cells identified as Kupffer cells (F4/80+, CD31+, blue bars), or endothelial cells (F4/80-, CD31+, red bars)
Opportunities for training and professional development: Nothing to report

Dissemination to communities of interest: Nothing to report

Plans for next reporting period:

- Assess biodistribution and gene silencing efficacy of priority bioconjugate-sdRNA formulations depicted in Figure 9.
- Encapsulate Chol-sdRNA into glucan shells and evaluate gene silencing in macrophages.
- Choose targets and identify effective siRNA sequences for Kupffer cell or hepatocyte silencing and synthesize appropriate sdRNAs for these targets.
- Initiate in vivo studies in rodent NASH and alcoholic fatty liver disease models towards the ultimate goal of gene silencing key gene targets and alleviating liver inflammation.

4. Impact:

Impact on principle discipline: Nothing to report

Impact on other disciplines: Nothing to report

Impact on technology transfer: Nothing to report

Impact on society: Nothing to report

5. CHANGES/PROBLEMS

This project continues to focus on the original goal of reducing the delivery vehicle for siRNA gene silencing from a 3 component system to a 2 component system. We also continue to aim to then use a newly developed 2 component system for siRNA delivery and gene silencing to alleviate fatty liver and non alcoholic and alcoholic steatohepatitis. Thus our goals remain the same, the overall approach remains the same, the way we are testing new vehicles for siRNA delivery remains the same and we are making significant progress towards those goals. We have changed the original chemistries of the vehicles we are testing based on our finding that low pH is required for the original weak base-modified glucan shells to effectively bind the siRNA, such that physiological pH is not optimal. Thus the new 2 component system we are employing attaches the moiety necessary for entry into the cell (e.g., cholesterol) onto the siRNA rather than onto the glucan shell, as originally envisioned. The modified cholesterol-siRNA (a single molecule) can then be encapsulated into the glucan shell, providing a 2 component system as originally proposed for our project.

We continue to use a strategy whereby the new 2 component chemistries that we develop are tested in vitro for silencing efficacy, tested in vivo for bio-distribution, then tested in vivo for gene silencing efficacy and then tested in vivo for ability to alleviate the disease indication (liver inflammation). We are in the process of executing this logical progression for novel (1 component and 2 component) siRNA delivery vehicles in this project, and remain optimistic that we can ultimately achieve the goal of proof of effectiveness of siRNA delivery to alleviate alcoholic and/or NASH by the end of this 3 year project.

We have successfully achieved Milestone 1 in our original SOW in that we have identified a siRNA vehicle structure (hydrophobic tail conjugated onto a chemically modified siRNA, as shown in Figure 9) that will be our priority formulation prototype as we move forward. As shown
in the accomplishment section, the cholesterol-siRNA prototype that we have already employed shows remarkable liver distribution from a subcutaneous injection. When injected alone (without encapsulation into glucan shells), this construct is directed to liver and is able to distribute to multiple cell types—a bonus and remarkable result. Our plan is to encapsulate that prototype into the glucan shells that will then be able to selectively target the siRNA to Kupffer cells. The change we have made in the chemistries of our priority prototype from the original plan (which was to modify the glucan shell rather than the siRNA molecule itself, as we are now doing) has thus put us into a much more versatile position for two reasons:

1. The hydrophobic moiety-modified siRNA constructs that we now have as our major priority can also be used alone (without encapsulation) to target multiple cells in liver and therefore be developed against other gene targets that can be effective in reducing inflammation and fibrosis. Upon encapsulation into glucan shells, we anticipate being able to target macrophages specifically, as we have already shown that is the case for GeRPs. Thus this new chemistry gives us greater flexibility as we proceed towards therapeutic efficacy.

2. The hydrophobic moiety-modified siRNA constructs that we now have as our major priority can be injected subcutaneously—a huge advantage over intravenous injection from a clinical perspective. As we have detailed in our progress report (Figures 10-12), subcutaneous injection of the cholesterol-siRNA construct is rapidly taken up by the liver in a variety of cell types. Thus, we are in position to exploit this more favorable injection route in our future development plans. When encapsulated by glucan shells, however, we will still need to use the intravenous route as originally proposed.

While we have focused almost exclusively in the fourth quarter of the project year 1 on subcutaneous injections of the Cholesterol-siRNA construct into mice (Figures 10-12), we have had to focus on bio-distribution of this new chemistry in vivo prior to testing gene silencing in vivo. This is necessary to be sure we know where the construct distributes upon in vivo administration. Then we will encapsulate into glucan shells and determine biodistribution during the next period, prior to then testing efficacy in gene silencing and alleviation of liver inflammation. Thus, we are delayed by a few months in testing gene silencing in vivo of these new constructs.

6. PRODUCTS: Nothing to report

7. PARTICIPANTS and OTHER COLLABORATING ORGANIZATIONS

Individuals who worked on the project:

Name: Michael P. Czech
Project Role: PI
Researcher Identifier (e.g. ORCID ID): NIH/eBRAP ID: MPCZECH
Nearest person month worked: 2.4

Contribution to Project: As PI Dr. Czech directed, designed and analyzed research.

Name: Adilson Guilherme
Project Role: Asst. Professor
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 8
Contribution to Project:  Dr. Guilherme designed and analyzed research, and participated in in vitro and animal experiments.

Name: David Pedersen
Project Role: Post Doc
Researcher Identifier (e.g. ORCID ID): NIH ID DPEDERSEN
Nearest person month worked: 12
Contribution to Project: Dr. Pedersen directed and participated in animal and in vitro studies of GeRp and sdRNA formulations.

Name: Yufei Shen
Project Role: Post Doc
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 3
Contribution to Project: Dr. Shen designed and synthesized GeRP formulations and carried out in vitro assessments.

Name: Sarah Nicoloro
Project Role: Res. Associate
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 3
Contribution to Project: Ms. Nicoloro provided technical assistance on all aspects of this project.

Changes in PI current support: Nothing to report

Other Organizations: Nothing to report

8. SPECIAL REPORTING REQUIREMENTS: N/A

9. APPENDICES: N/A