Pennsylvania State University

Annual Progress Report: 2011 Nonformula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Nonformula Grant Overview

The Pennsylvania State University received $1,000,000 in nonformula funds for the grant award period June 1, 2012 through November 27, 2014. Accomplishments for the reporting period are described below.

Research Project: Project Title and Purpose

Therapeutic Delivery of siRNA Using Calcium Phosphate NanoJackets for Improved Cancer Treatment – Currently molecular-based therapeutic approaches, including siRNA, are limited by poor cellular uptake and cellular degradation. The design and engineering of delivery systems that protect and target molecular-based therapeutics is a critical unmet need in the arsenal of cancer-fighting agents. Nanotechnology offers the promise of enhancing cancer cell uptake while protecting the siRNA cargo during systemic delivery. Penn State University has engineered a non toxic, biocompatible, uncharged calcium phosphate nanoparticle that has proven efficacious for delivering siRNA to cancer. In collaboration with Keystone Nano and Calvert Laboratories, the physical, chemical and biological properties of these nanocolloids will be evaluated. These commercialization studies will provide the data for a preclinical package that will support an IND application to the FDA.

Anticipated Duration of Project

6/1/2012 – 11/29/2014

Project Overview

The research objective is to commercially develop improved cancer treatments that utilize biocompatible, bioresorbable and bioneutral calcium phosphate nanoparticles, known as NanoJackets, for systemic therapeutic delivery of small interfering ribonucleic acid (siRNA) molecules. However, the ability to effectively deliver siRNAs for cancer treatments has proven challenging, particularly for systemic administration. The ability to concentrate siRNAs to achieve therapeutic threshold concentrations in tumors remains difficult. Non-toxic nanoscale delivery modalities may offer the best chance to achieve therapeutic dosing of siRNAs within tumors.

As proof of concept, we have developed and demonstrated the biological efficacy of siRNA

NanoJacket prototypes targeting the 3140A>G point mutation in the human PI3K gene that is over-expressed in a cohort of breast cancer patients with poor outcomes. This approach is distinct from competitor technologies in two main ways. First, the use of calcium phosphate particles that can be produced at the 100nm scale alleviates the toxicity and limited biodistribution profiles observed with current nano-delivery technologies. Second, the strategy to design siRNA oligonucleotides for selected cancer-causing mutations provides an additional layer of specificity that will minimize off-target side effects.

To support initial product development of the 3140A>G PI3K siRNA NanoJackets, the overall goal of the proposed project is to enable an investigational new drug (IND) application to the FDA for regulatory review. In order to achieve the next stages of commercialization for the 3140A>G PI3K siRNA nanoproduct, the following specific aims are proposed:

1. Selection of optimal siRNA NanoJacket formulation based on in vivo efficacy
2. Optimization of manufacturing to produce material for chemistry, manufacturing and control (CMC) characterization and preclinical testing
3. Evaluation of dose-defining in vivo studies
4. Completion of pivotal GLP preclinical studies
5. Compilation of siRNA NanoJacket IND package for FDA review

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Mylisa Parette, PhD; Carly Carter, PhD – employed by Keystone Nano, Inc.

Expected Research Outcomes and Benefits

Cancer is a disease that results from genetic mutations that cause aberrant cell survival and growth. The vast majority of current chemotherapeutic treatments utilize cytotoxic pharmacological or radioactive compounds to kill cancerous cells. However, virtually all of these treatments result in toxic side effects, mostly due to the nonselective mechanisms of pharmaceutical action and poor biodistribution profiles. In fact, severe side effects often limit the dosage that patients can receive. For many cancers, this leaves severely ill patients with little choice but to endure widespread toxicity for small gains in therapeutic efficacy.

The present grant focuses on another approach, the delivery of molecular-based therapeutics that

target mutated oncogenic proteins, over-expressed preferentially in cancer cells. The overall expected outcome of this proposal is to complete the preclinical evaluation of a nontoxic, biocompatible nanoscale delivery system for siRNA. Although the focus of the proposed project will center on NanoJackets delivering siRNA designed for the 3140A>G point mutation in the PI3K gene, a common breast cancer mutation, the encapsulation of siRNA within NanoJackets is sequence-independent. So while this project will provide for the commercialization of a defined RNAi-based breast cancer therapy, it will also serve as proof of concept for a platform siRNA delivery technology. Further, with the increasing use of pharmacogenetics to identify specific mutations in primary tumor biopsy samples, siRNA NanoJackets could provide a customized therapeutic product that targets each patient's specific cancer-causing mutations. Thus, the true benefit of the research program is the commercialization of a platform delivery system that allows for the realization of personalized medicine.

Since siRNA NanoJackets can be utilized as a platform technology, successful funding and completion of this project will likely lead to considerable revenue, growth and job creation within PA as well as improve the health status of Pennsylvanians. In addition, this will serve as an effective model by which to translate technologies developed in Universities to commercial nanomedical products, which could promote further commercialization of University technologies.

Summary of Research Completed

Specific Aim 1-Selecting optimal siRNA NJ formulation based on in vivo efficacy

Task 1a-Selecting optimal siRNA sequences for knockdown of PI3K *mutations in breast cancer cells*
This task was completed as detailed in the annual report for FY 2012-13.

Task 1b-Evaluating PI3K *siRNA NJ prototypes in vitro:*
Although siRNA NanoJacket formulations were ineffective *in vitro*, as described in our previous report, the effect of the P19 and H6 siRNA sequences on PI3KCA and HER2 protein expression was measured to confirm the RNAi mechanism of action. *In vitro* protein knockdown was quantitated after 50 nM P19 (PI3KCA 3140 A>G targeted), wt P19, H6 (wt HER2 targeted) or 60/40 P19/H6 siRNA was transfected via Lipofectamine into both cancerous (HCC1954) and normal (MCF10A) cells. The normalized knockdown data, Figure 1, shows statistically significant 99% knockdown of HER2 by both H6 and 60/40 P19/H6 in HCC1954 cancer cells. In addition the P19 siRNA designed to target a single point mutation in PI3KCA showed selectivity, significantly decreasing PI3KCA expression in cancerous cells to only 60% of untreated levels, while having no effect on normal cells.

Task 1c- Comparing siRNA NJ prototypes in vivo:
As previously described, the P19/H6 siRNA NanoJackets were created and tested in a xenograft tumor model of human breast cancer (HCC1954) and induced a significant decrease in tumor growth. Recently, we have been able to analyze the tumor lysates from that study for PI3KCA and HER2 expression, Figure 2, showing 97% knockdown of both proteins, which is

considerably higher than any published values insofar as we know.

Specific Aim 2-Optimizing manufacturing to produce material for chemical manufacturing and controls characterization and preclinical testing

Task 2a-Develop optimal large scale manufacturing method
In order to produce larger quantities of siRNA NanoJackets to support further in vivo experimentation, it was necessary to identify suppliers that were capable of providing quality PEG and siRNA, at reasonable prices with relatively short lead times. In an effort to maintain our scaled up manufacturing methods a company was contracted to synthesize large-scale (gram) quantities of our siRNA due to their reasonable pricing and reputation as a respected supplier of clinical oliogonucleotides. Initial results showed that the siRNA from this supplier did not form NanoJackets of expected size and stability. Through extended experimentation and using a novel assay developed in house, the siRNA was shown to be contaminated with a surfactant that originated from the suppliers oligonucleotide purification process. A subsequent batch of siRNA, conforming to more stringent purity specifications demonstrated incorporation into NanoJackets as expected (Figure 3). This material was used to produce NanoJackets to be tested *in vivo* for efficacy, pharmacokinetics and maximum tolerated dose studies (Specific Aim 3, Tasks a, b and c). However, throughout the process of manufacturing large batches of NanoJackets, the quality of the NanoJackets began to degrade. Interestingly, old batches of siRNA from a previous supplier (stored on the bench for 18 months) were still capable of producing high quality NanoJackets. The faulty material obtained from the initial supplier caused the failure of an *in vitro* efficacy, an *in vivo* pharmacokinetic study, and an *in vivo* maximum tolerated dose study (Tasks 3a, 3b and 3c). In an effort to have a more consistent manufacturing process, more stringent specifications on the starting materials have been adopted. We have retained the services of Dharmacon, who, while at considerably higher cost, has a proven capability to produce both the quality and quantity of siRNA amenable to our manufacturing processes. The NanoJackets to complete Specific Aim 3 have been successfully manufactured using RNA obtained from Dharmacon.

Laysan Bio and NOF have been identified and verified as suppliers of cGMP quality PEG of varying lengths, with lead times ranging from days to weeks depending on the quantity and type of PEG needed.

Each of the 4 steps (Figure 4) in the NanoJacket formation protocol was modified to optimize manufacturing for large-scale production. The conjugation reaction, step 1, was successfully scaled up nearly 40-fold, and the reaction efficiency was increased by 25% by utilizing more consistent temperature control. Due to the increased reaction scale, modifications to the purification process were necessary. Although larger scale reactions were not compatible with the original ultracentrifugation processing, a two-step desalting process in combination with centrifugation at 15000 x g to purify the conjugated siRNA is feasible. We have also developed a new small-scale purification protocol via desalting resin and molecular weight cut-off filters that allows us to purify the PEGylated siRNA in 50% of the time, and are currently testing the protocol on a large scale. Additionally, NanoJacket formation reactions (step 3) were scaled up 50-fold and subsequent NanoJacket purification processes were linearly scaled, decreasing

purification time by 99%. In combination, the changes to our manufacturing processes have more than doubled our production efficiency from 40 mg of NanoJackets in 6 weeks, to 85 mg of NanoJackets in 4 weeks.

As we reported in the previous annual report, the encapsulation efficiency for siRNA Nano Jackets is 20%. Therefore, a novel method of recycling the uncaptured siRNA to facilitate further NanoJacket production was developed. That process has been further improved via molecular weight cut off filters so that we can now recycle the unincorporated material, decreasing the time required for recycling by 92%. We have demonstrated successful incorporation of the recycled siRNA into NanoJackets (data not shown). This development combined with our more efficient scaled up processing decreases our manufacturing time while making the process more cost effective.

Task 2b- CMC Characterization:
We have continued to monitor the stability of NanoJackets over time while stored at room temperature and have not seen a change in particle size dispersion (Figure 5A). Additionally, the NanoJackets were incubated in RPMI media with 20% FBS for 24 hours at 37°C without degrading (Figure 5B).

Specific Aim 3- Evaluation of dose-defining in vivo studies
Task 3a-Pharmacokinetics/Biodistribution
We have adapted a previously published protocol utilizing qPCR to ascertain both pharmacokinetic and biodistribution information rather than using radiolabeld siRNA as originally proposed. The qPCR method was developed because the quantity of stable radiolabeled nucleotides required for our studies was cost prohibitive. Despite initial quotes to incorporate a radiolabel on one end of the siRNA sequence, that chemistry would not have been stable under our manufacturing conditions. To facilitate qPCR amplification, total RNA is extracted from serum or tissue samples and RT-PCR is used to amplify and quantitate the siRNA in a given sample. Preliminary data from a qPCR experiment where siRNA was spiked into tissue samples demonstrates that the technique is a viable option to provide us with data regarding PK and biodistribution (Figure 6). As mentioned above, a PK study was attempted but failed due to poor quality siRNA obtained from the initial siRNA supplier. We have completed the manufacturing of the material required to repeat the failed study.

Task 3b-Dose range finding studies
Phase one of the maximum tolerated dose study was conducted using 4 female Sprague Dawley rats each dosed at 0.5, 1, 1.5, 2.5 and 5 mg/kg NanoJackets consecutively at four hour intervals. Toxicity was not observed over the course of 24 hours, although some lethargy was noted after the 1.5 and 2.5 mg/kg doses. Phase 2 of the maximum tolerated dose study has begun.

Task 3c- Efficacy in murine models using optimal dose and schedule
The material required for the efficacy study described in the original proposal has been prepared.

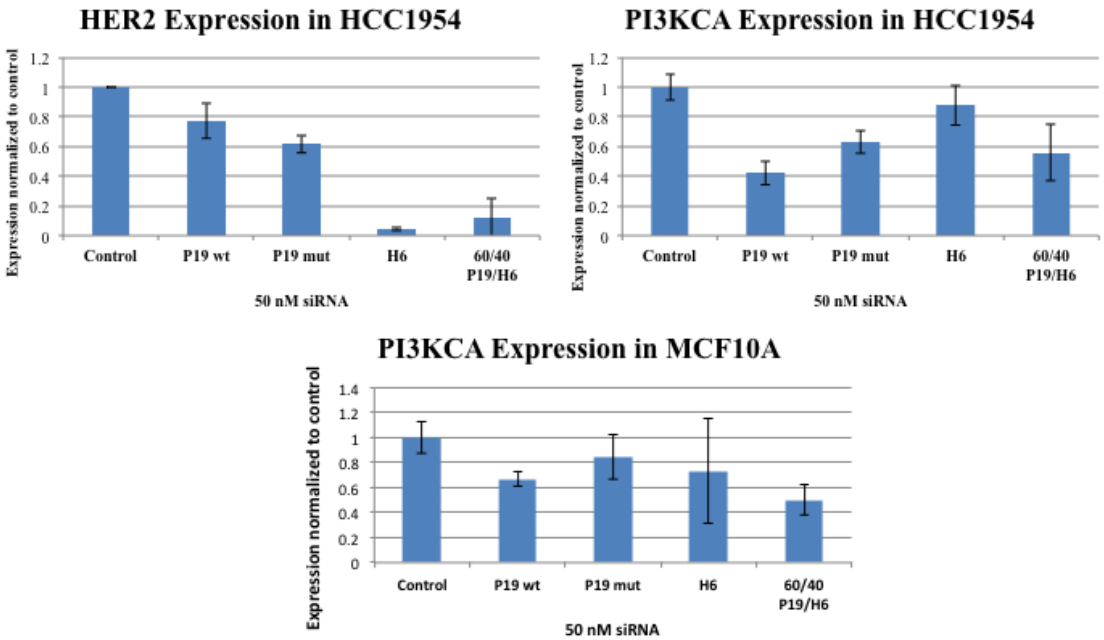

Figure 1. HER2 and PI3KCA knockdown after transfection of 50 nM siRNA in either normal breast cells (MCF10A) or cancerous breast cells (HCC1954). Control is untreated cells.

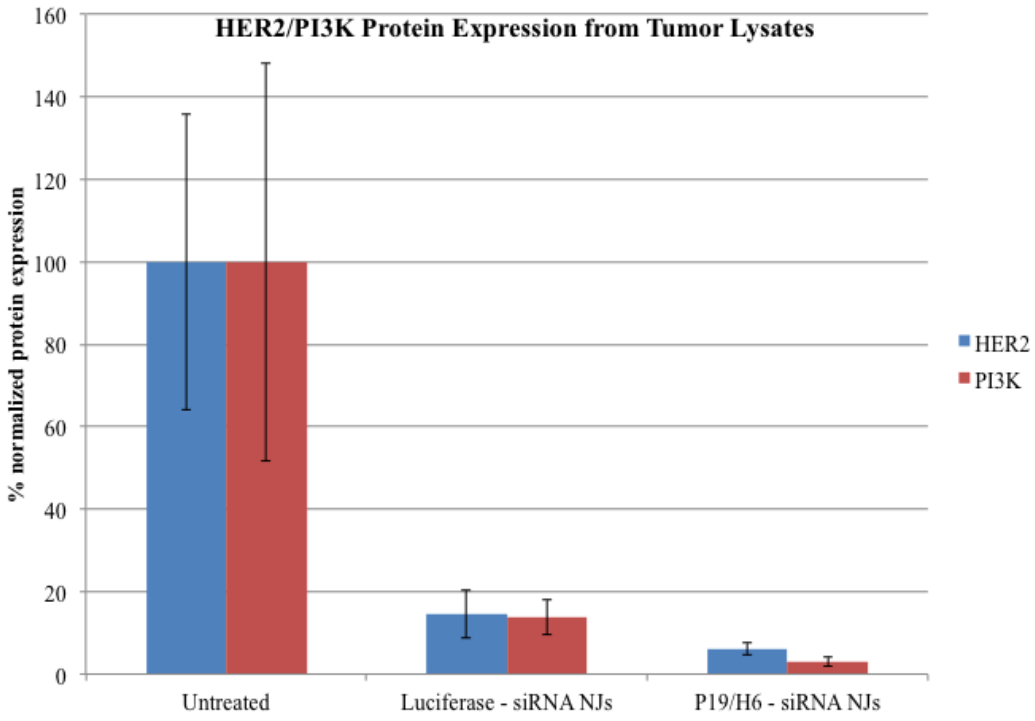

Figure 2. Her2 and PI3KCA knockdown in tumor lysates from untreated, luciferase NanoJacketed siRNA treated or P19/H6 NanoJacketed siRNA treated mice.

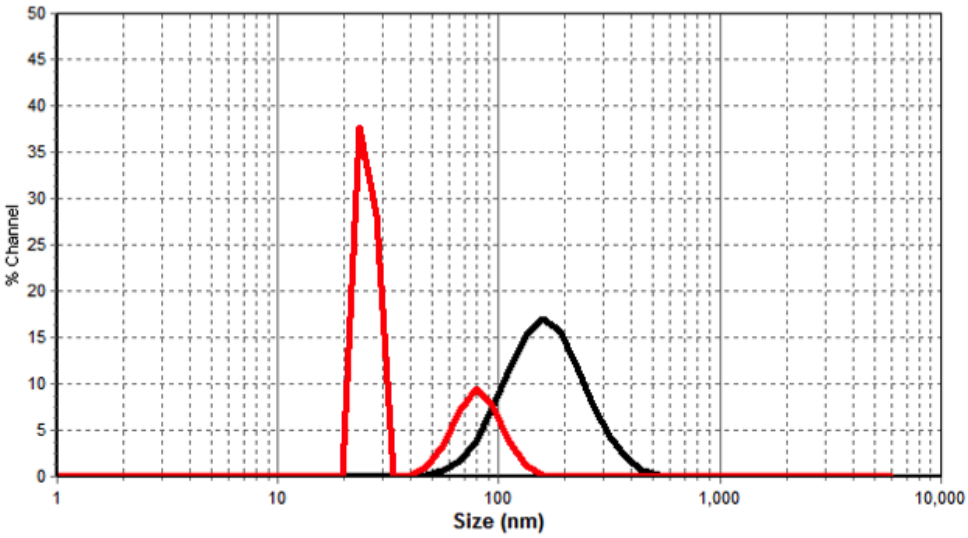

Figure 3. Dynamic Light Scattering spectra of NanoJacketed siRNA made from Avecia siRNA in January 2014 (black) and May 2014 (red) shows degradation over time.

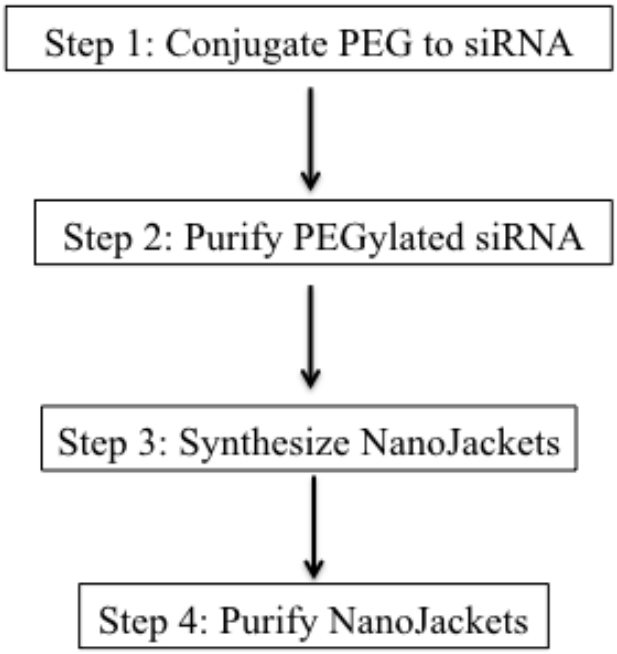

Figure 4. Flow Chart of NanoJacket manufacturing steps.

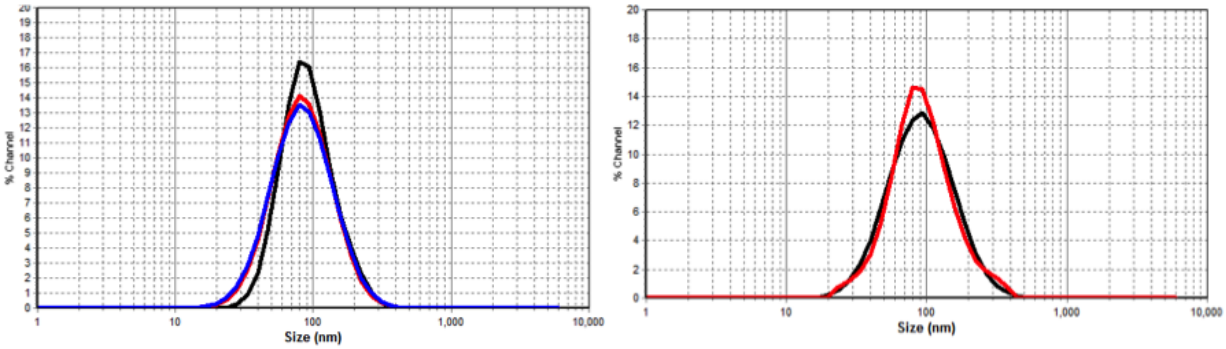

Figure 5. Dynamic Light Scattering spectra of (**A**) 25% PEGylated NanoJacketed siRNA after 2 days (black), 1 month (red) and 15 months (blue) store at room temperature and (**B**) NanoJackets in water (black) and after incubation in RPMI with 20% FBS for 24 hours at 37 °C (red).

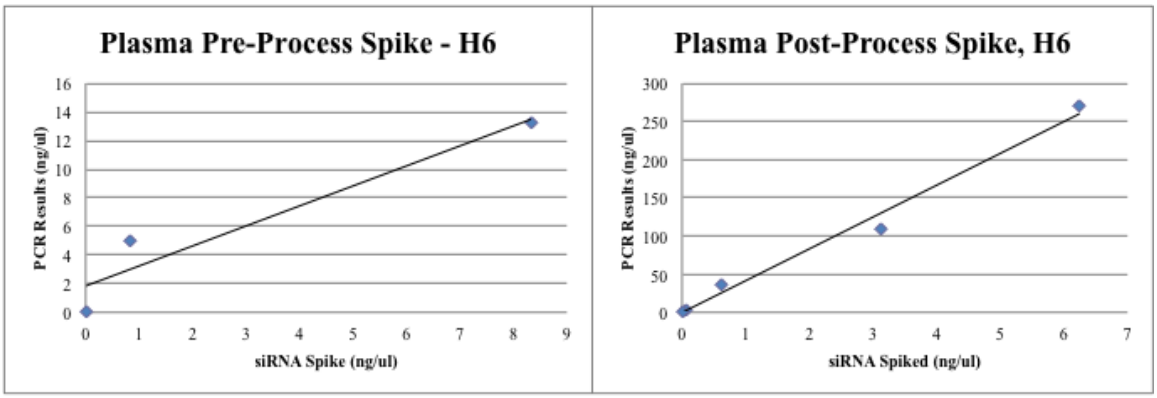

Figure 6. qPCR data obtained from 60/40 P19/H6 siRNA spiked into plasma samples pre (R^2=0.92) and post (R^2= 0.98) total RNA extraction protocol.