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PRINCIPAL INVESTIGATOR: Dr. Akhil Srivastava

CONTRACTING ORGANIZATION: University of Oklahoma, Board of Regents of the University of OK

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project was focused on development of novel theranostics for simultaneous treatment, measuring treatment response and diagnosis of lung cancer. Current chemotherapeutic interventions are though effective in inhibiting cancer cell growth under in vitro condition but loses their potency when administered to patient's body. Absence of an efficient drug delivery system is the main cause of failure of chemotherapy. In addition, Lung cancer is often detected at late stages and current modalities of imaging are also not very precise. Hence the main objective of this project was to develop an efficient drug delivery vehicle, which can deliver drug precisely to the tumor site. In addition this vehicle also possessed ability to imaging using Magnetic Resonance Imagining (MRI) and is supposed to give precise imaging of tumor. The information generated in this project will serve as a proof of concept for the purposed technology and will open scope of future studies needed to develop it as a method that will enable clinicians to design precise and personalized treatment regimens.

15. SUBJECT TERMS

Exosomes, Lung cancer, Drug delivery Theranostics, Chemotherapy, MRI, Precision medicine.

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An abstract shall be provided in Block 14 and shall state the purpose, scope, and major findings and be an up-to-date report of the progress in terms of results and significance. Abstracts will be submitted to the Defense Technical Information Center (DTIC) and shall not contain proprietary information. Subject terms are keywords that may have been previously assigned to the proposal abstract or are keywords that may be significant to the research.

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INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

1.

This project was focused on development of novel theranostics for simultaneous treatment, measuring treatment response and diagnosis of lung cancer. Current chemotherapeutic interventions are though effective in inhibiting cancer cell growth under *in vitro* condition but loses their potency when administered to patient's body. Absence of an efficient drug delivery system is the main cause of failure of chemotherapy. In addition, Lung cancer is often detected at late stages and current modalities of imaging are also not very precise. Hence the main objective of this project was to develop an efficient drug delivery vehicle, which can deliver drug precisely to the tumor site. In addition this vehicle also possessed ability to imaging using Magnetic Resonance Imagining (MRI) and is supposed to give precise imaging of tumor. The information generated in this project will serve as a proof of concept for the purposed technology and will open scope of future studies needed to develop it as a method that will enable clinicians to design precise and personalized treatment regimens.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Exosomes, Lung cancer, Drug delivery Theranostics, Chemotherapy, MRI, Precision medicine.

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1(specified in proposal)
Major Task 1
Exosomes from lung epithelial cell lines (BEAS2B or NHBE, purchased from ATCC) will be primed with hsa-miR-124-3p by physical loading such as electroporation or incubation and the successful loading will be evaluated by qPCR.
In parallel to above step Super Paramagenetic Iron Oxide Paramagnetic Nanoparticle (SPION) will be conjugated with Cisplatin (CDDP) and will be decorated with –anti B7-H4 scFv. Finally, the nanoparticle complex created will be loaded to miRNA primed exosomes. The B7-H4-Exo-NP-CDDP complex will then be characterized by measuring zeta potential, Electron microscopy, Inductively coupled plasma mass spectrometry (ICP-MS), nanotracker analysis and OPDA assay
Target time: 1-2 months from start date of project. Completion time: 1 month from the start of project, few experiments are still pending. Percentage of completion: 85% completed; Addition of targeting molecule (scFv-B7H4) is still pending.
Major Task 2

Cell uptake study of B7-H4-Exo-NP-CDDP by NSCLC cells (A549, HCC 827 purchased from ATCC) and normal cells (BEAS-2B and NHBE purchased from ATCC). We will also test the complex on Mouse lung cancer cell lines (LLC) and normal cells (BNL1-MEA) All cells will be purchased from ATCC. MRI imaging Drug release kinetics and therapeutic efficacy study DNA damage analysis by comet assay and western immunoblottings
Submission of animal protocol to institute's IACUC and IRB committee
Target time: 3-7 months from start date of project. Completion time: 4 month from the start of project, few experiments are still pending. Percentage of completion: 75% completed; additional experiments pending are testing with more lung cancer cell lines, and experiments with normal cell lines.
Major Task 3
Local IRB/IACUC Approval and animal protocol will be forwarded to ACURO for further approval
The impact of B7-H4-Exo-NP-CDDP will be studied for T cell proliferation. T cell surface markers will be studied for monitoring the change in T cell morphology. Flow cytometry assay and MTT assay will be used to measure the cell death or proliferation. Jurkat cells will be used for this study and will be purchased from ATCC. *In vivo* studies to validate the delivery of therapeutics through B7-H4-Exo-NP-CDDP delivery system and estimate its effect in nude mice
In vivo studies to validate the delivery of therapeutics through B7-H4-Exo-NP-CDDP delivery system and estimate its effect in nude mice and immunocompetent mouse (n=20 nude and n=20 immunocompetent mouse will be used in this study). Tumor growth will be determined to determine the effective ness delivery and MRI imaging will be done to see the efficacy of diagnostic potential of the complex. Additional molecular assays will also be performed (immunoblotting, staining and PCRs) on tissues after euthanizing the animal after termination of experiment to provide additional evidences of the effectiveness of the complex.
Target time: 7-12 months from start date of project. Percentage of completion: 0%; This task is yet to be completed for which project extension is requested.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Please see **Annexure 1**

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities. .

One laboratory technician was trained exosome isolation, cell culture and other regular laboratory techniques.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We have now shown as a proof of concept the feasibility of Exo-NP-CDDP complex. Our future plans are to add targeting moiety and do the in vivo studies in lung cancer mouse models.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We envision that the theranostic we are developing will bring changes in modalities of drug delivery and imaging of tumors. This will help in closely monitoring the treatment response in patients against a given therapeutic.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

There has been no significant changes.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

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. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

1. Attended as participant ISEV-MRS joint meeting on Extracelleular vesicles in cancer, 2-4 August 2019, Nashville, TN.
2. Akhil Srivastava, Narsireddy Amreddy, Rebaz Ahmed, Meghna Mehta, Daniel Zhao, Anupama Munshi and Rajagopal Ramesh, **Exosomes as Theranostics in Lung Cancer.** Abstract submitted to END2Cancer Conference, November 20-22, 2019 Oklahoma City, OK.

Website(s) or other Internet site(s)

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

Technologies or techniques

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

Inventions, patent applications, and/or licenses

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

Other Products

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Rebaz Ahmed
Project Role:
Nearest person month worked: 1
Contribution to project:

Name: Rajagopal Ramesh
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution to the project:

Name: Akhil Srivastava
Project Role: Principal Investigator
Nearest person month worked: 3
Contribution to the project:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

* Not applicable (annual final report)

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

University of Oklahoma Health Sciences Center,
Oklahoma Medical Research Foundation
University of Oklahoma
Oklahoma State University

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Annexure 1

4. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Task 1

1. Major activities: Creation and characterization of B7H4-Exo-NP-CDDP vehicle
2. Specific objectives:
    i. Standardization and characterization of exosomes from cell culture media (supernatant).
    ii. Evaluation of baseline expression of B7H4 protein and B7H3 mRNA in lung cancer cell lines.
    iii. Construction and characterization of Exo-NP-CDDP complex.

3. Significant results: Prior to start of the specific objectives proposed in the funded grant, the protocol for exosome isolation from normal lung fibroblast MRC9 cells was optimized following the regulations of International Society of Extracellular Vesicles (ISEV) and the presence of exosomes confirmed in our sample preparations. Transmission electron microscopy (TEM) study showed presence of typical double membrane cup shaped vesicles as described in literature for exosomes (Fig. 1A). The size and shape of the isolated exosomes were further measured by Nano tracker device called NanoSight N300. The screen grab from the isolations (Fig. 1B) and the size and number distribution graph (Fig.1C) also confirmed the presence of exosomes. Lastly, western blotting showed presence of exosome marker CD63 in the exosome preparation (Fig. 1D).

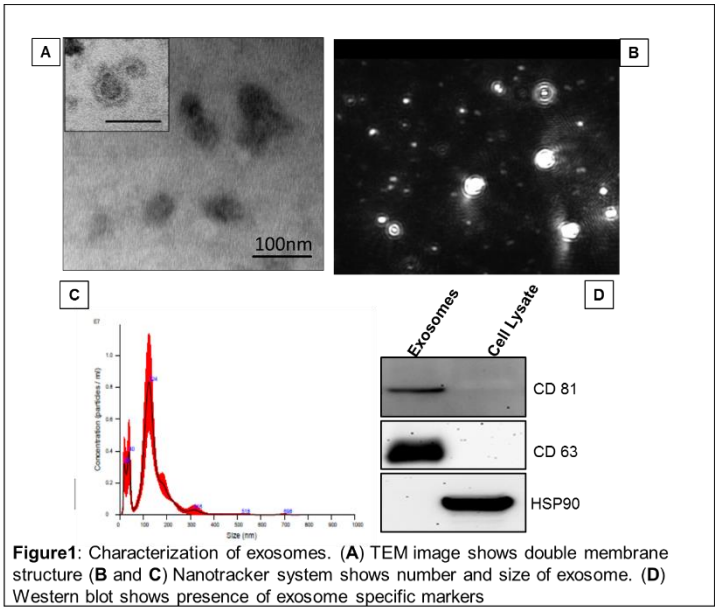

Figure1: Characterization of exosomes. (**A**) TEM image shows double membrane structure (**B** and **C**) Nanotracker system shows number and size of exosome. (**D**) Western blot shows presence of exosome specific markers

4. Next, for incorporating scFV-B7H4 in the exosome complex, the baseline expression of B7H4 protein was assessed in a panel of human lung cancer cell lines and two normal cell

lines (Fig. 2). On the basis of our data, we chose to use A549 lung adenocarcinoma cell line and MRC9 lung normal fibroblast cell line for our studies. Next, we analyzed for the immune checkpoint B7H3 mRNA and hsa-miR143-3p micro (mi)RNA expression levels in A549 and MRC9 cells respectively (Fig. 3A&B). On the basis of the preliminary data obtained, exosomes from MRC9 cells were isolated, purified, and used for preparing theranostic complex. For this purpose, MRC9-derived exosomes were primed with has-miR124-3p and followed by loading of SPION (Super Paramagnetic Iron Nanoparticles which will be referred as NP in text) conjugated with anti-cancer drug 'cisplatin' (CDDP) onto the exosomes by simple incubation. Successful generation of the theranostic complex was confirmed by TEM imaging (Fig. 4A) and incorporation of SPION nanoparticle was confirmed by measuring through Inductively Coupled Plasma Mass Spectrometry (ICPMS) and Magnetic Resonance Imaging (MRI) (Fig. 4B; Table 1). Finally, OPDA assay demonstrated the amount of CDDP drug loaded into the exosome complex. The prepared complex was labeled Exo-NP-CDDP.

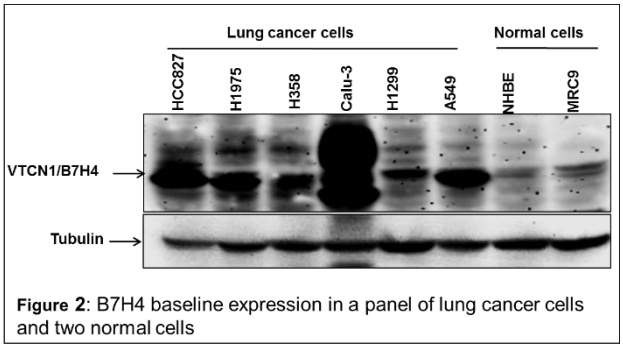

Figure 2: B7H4 baseline expression in a panel of lung cancer cells and two normal cells

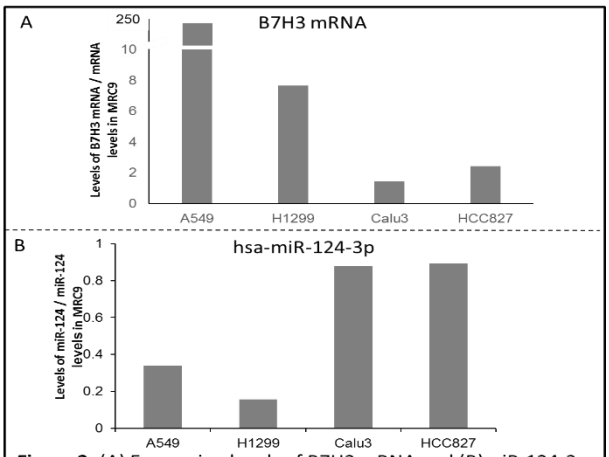

Figure 3: (A) Expression levels of B7H3 mRNA and (B)miR-124-3p in lung cancer cells compared against respective levels in MRC9 cells

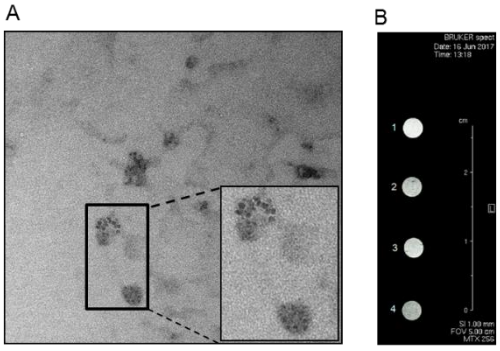

Figure 4: Successful incorporation of SPION on exosomes was confirmed by (**A**) TEM imaging and (**B**) MRI analysis

Table1: Increase in T2 values indicates successful incorporation of SPIONS on Exosomes

Sample	T2 values	R2 vales
Control	105.09	9.51
SPION 100ug	79.09	12.64
Exo-NP-CDDP 4.1ug	102.81	9.72
Exo-NP-CDDP 8.2ug	104.03	9.61

5. Incomplete goals: At present, sufficient quantities of scFv-B7H4 have not been obtained from the commercial vendor whom we had contracted. As a result we have not been able to add scFv-B7H4 to the exosome complex. We are currently exploring other vendors and avenues for acquiring sufficient quantities of scFv-B7H4. Nevertheless, to prove the concept

I proceeded with non-targeted version of complex and have confirmed the successful creation of this complex.

6. Minor changes: Some minor changes made to the proposed are that we have used MRC9 normal lung fibroblast cell line instead of using NHBE or BEAS2B- the normal cell lines. The reason for using MRC9 over NHBE and BEAS2B was easy to grow and maintain them for use in multiple experiments.

Major Task 2

1. Major activities: to study cellular uptake and therapeutic effect of drug delivered by B7-H4-Exo-NP-CDDP complex under *in vitro* condition
2. Specific objectives:
   i. To show the successful uptake of Exo-NP-CDDP complex by the recipient cells
   ii. Show the therapeutic effect of Exo-NP-CDDP complex in the recipient cancer cell

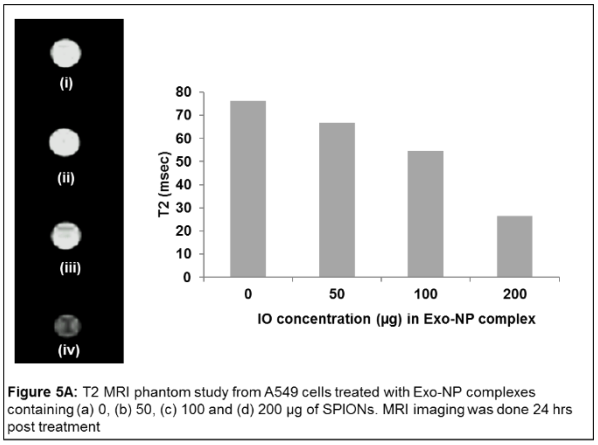

Figure 5A: T2 MRI phantom study from A549 cells treated with Exo-NP complexes containing (a) 0, (b) 50, (c) 100 and (d) 200 µg of SPIONs. MRI imaging was done 24 hrs post treatment

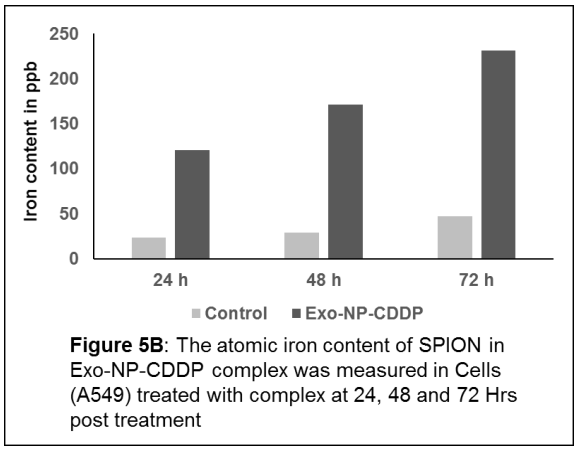

Figure 5B: The atomic iron content of SPION in Exo-NP-CDDP complex was measured in Cells (A549) treated with complex at 24, 48 and 72 Hrs post treatment

3. Significant results: Once the complex Exo-NP-CDDP complex was generated, I studied and showed successful uptake of Exo-NP-CDDP complex by recipient tumor cells. A549 lung tumor cells were treated with Exo-NP-CDDP and analyzed for uptake by MRI, ICPMS and Prussian blue staining of cells after adding the complex (Fig. 5 A, B&C). Further, therapeutic effect of complex on recipient A549 was demonstrated by cell viability assay (Fig. 6).

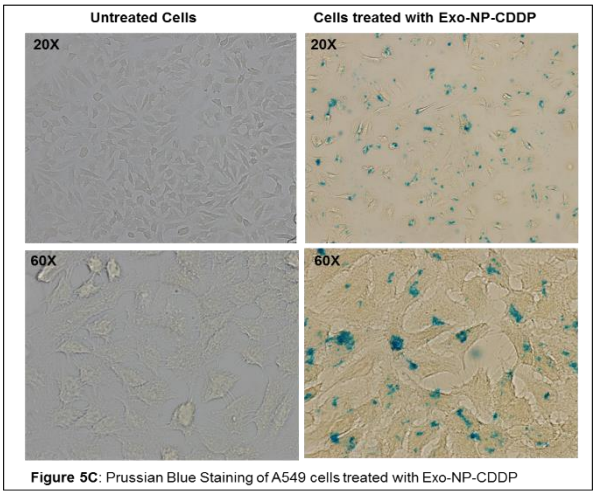

Figure 5C: Prussian Blue Staining of A549 cells treated with Exo-NP-CDDP

Another target of the project was to submit IACUC protocol and receive approval which was also successfully completed and approval received from institutional IACUC office and from ACURO of DoD.

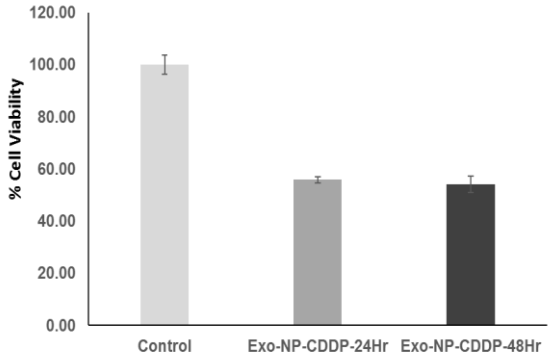

Figure 6: The therapeutic effect of Exo-NP-CDDP was observed by reduction in cell viability at 24 and 48 hrs post treatment. A549 cells were treated with Exo-NP-CDDP in this study

4. Incomplete goals: Experiments with mouse cell lines and normal cell line remains to be done. The experiments are currently being carried out and will be finished soon.
5. Minor changes: None

Major Task 3

1. Major activities: B7-H4-Exo-NP-CDDP delivery system is established for targeted delivery of anti-cancer therapeutics at cancer site *in vivo* condition.

2. Significant results: None

3. Incomplete goal: We need to finish this objective but due to delay in release of funds the project got a late start and we need to finish objectives of Major task 1 and 2 before I start this task.