Targeting Extracellular Matrix Glycoproteins in Metastases for Tumor-Initiating Cell Therapy

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12. DISTRIBUTION / AVAILABILITY STATEMENT

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14. ABSTRACT

The proposed research is a proof-of-concept study that focuses on testing a new cancer targeting strategy that aims at enhancing nanodelivery of drugs to osteopontin (OPN) that are often overexpressed in advanced prostate cancer cells and their microenvironment. To evaluate this strategy, lipid-based nanocarrier that targets OPN (i.e. OPN-LN) was developed, characterized and compared with non-targeting LN. This OPN-LN was used as the key platform to study the OPN targeting strategy. In the reporting period, OPN-LN was prepared by conjugation of OPN antibody onto the surface of lipid nanocarriers using SATA as the conjugation reagent. Our data show that the OPN-LN have good dispersion stability (no noticeable aggregation at 37 °C in 2 days) and regular morphology. When compared with non-targeting LN, OPN-LN were more efficiently taken up by PC-3M prostate cancer cells which were shown to be OPN expressing as indicated by Western blotting analysis. No significant increase in non-specific toxicity was observed after OPN antibody conjugation. In brief, OPN targeting apparently can improve the nanodelivery to OPN expressing prostate cancer cells. The impact on anticancer efficacy will be evaluated in the next reporting period.

15. SUBJECT TERMS

Osteopontin, prostate cancer, targeted delivery, nanomedicine

16. SECURITY CLASSIFICATION OF:

a. REPORT b. ABSTRACT c. THIS PAGE

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17. LIMITATION OF ABSTRACT

UU

18. NUMBER OF PAGES

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19a. NAME OF RESPONSIBLE PERSON

USAMRMC

19b. TELEPHONE NUMBER (include area code)

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1. **INTRODUCTION:**

The proposed research is a proof-of-concept study that focuses on testing a new cancer targeting strategy that aims at enhancing nanodelivery of drugs to the glycoproteins (e.g. osteopontin, OPN) that are often overexpressed in advanced prostate cancer cells and their microenvironment. The purpose is to establish this novel delivery strategy for effective and safe prostate cancer therapy. To evaluate this strategy, in the first stage, it was proposed that a nanocarrier that targets OPN will be developed and characterized, and this nanocarrier will be used for determining the feasibility of the proposed OPN-targeting strategy (i.e. Aim #1 – To determine the feasibility of OPN-targeted strategy for enhancing nanomedicine delivery to OPN-rich targets). In the second stage, this OPN-targeting nanocarrier will be loaded with an anticancer drug and the in vitro therapeutic activities against prostate cancer cells with TIC behaviors will be studied (i.e. Aim #2 – To study the therapeutic effects of OPN-targeted delivery on metastatic prostate cancer).

In general, the tasks listed under both aims are completed. A manuscript is being prepared and is close to getting submitted pending some final polishing. This is the FINAL report for this award.

2. **KEYWORDS:**

Osteopontin, prostate cancer, targeted delivery, nanomedicine
3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

  **Specific Aim 1:** To determine the feasibility of OPN-targeting strategy for enhancing nanomedicine delivery to OPN-rich targets.
  - Objective 1: Prepare and characterize OPN-targeting carrier (month 1-6) (completed in 12 months)
  - Objective 2: Evaluate the effect of decorating a nanocarrier with OPN-targeting moieties on OPN-binding (month 1-6) (completed in 12 months)
  - Objective 3: Evaluate the effect of OPN-targeting on nanomedicine delivery to cell culture (month 4-9) (completed in 12 months)

  **Specific Aim 2** To study the therapeutic effects of OPN-targeted delivery on metastatic prostate cancer
  - objective 1: Preparation of prostaspheres (month 1-6) (not completed)
  - objective 2: Evaluate the therapeutic effects of OPN-targeting system carrying a hedgehog pathway inhibitor (month 3-12) (not completed)

- **What was accomplished under these goals?**

  **Major activities and specific objectives:** In the funding period, we focused on completing the objectives of both Aims as listed in the SOW including

  1. **Aim 1:**
     - objective 1: Prepare and characterize OPN-targeting carrier
     - objective 2: Evaluate the effect of decorating a nanocarrier with OPN-targeting moieties on OPN-binding
     - objective 3: Evaluate the effect of OPN-targeting on nanomedicine delivery to cell culture
  2. **Aim 2:**
     - objective 1: Preparation of prostaspheres
objective 2: Evaluate the therapeutic effects of OPN-targeting system carrying a hedgehog pathway inhibitor

**Significant results:** The more significant results are summarized as below

1. **Specific Aim 1:** To determine the feasibility of OPN-targeting strategy for enhancing nanomedicine delivery to OPN-rich targets.

   **a. Preparation of Osteopontin(OPN)-targeting carrier (under Objective 1).** Lipid nanoparticles were prepared using a blend of biocompatible lipids and phospholipids such as triglyceride, triolein, DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), and/or PEG-DSPE (Polyethylene glycol-distearoyl-glycero-phosphoethanolamine). These nanoparticles were conjugated with OPN-antibody using SATA as conjugation agent. The OPN-targeting lipid nanoparticles (OPN-LN) showed no visible aggregation and precipitation at 37 °C in 72 hours. OPN-LN were further characterized and the results are shown as below.

   **b. Dynamic light scattering: Size, PDI, zeta potential (under Objective 1).** The average size, polydispersity (PDI) of size and zeta potentials of LN before and after conjugation with OPN-antibody are measured using dynamic light scattering technique (Zetasizer, Malvern, UK) and shown below. In addition, in preparation of the works under Specific Aim 2 we also attempted to encapsulate a hedgehog pathway inhibitor cyclopamine (CP), and the data are as follows:

<table>
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<tr>
<th></th>
<th>Average diameter (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
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</thead>
<tbody>
<tr>
<td>Blank LN, no OPN-antibody</td>
<td>149.4</td>
<td>0.196</td>
<td>-20.0</td>
</tr>
<tr>
<td>Blank LN, +OPN-antibody coating</td>
<td>154.3</td>
<td>0.174</td>
<td>-18.2</td>
</tr>
<tr>
<td>LN-loading CP, no OPN-antibody</td>
<td>161.7</td>
<td>0.181</td>
<td>-28.7</td>
</tr>
<tr>
<td>LN-loading CP, +OPN-antibody</td>
<td>169.8</td>
<td>0.198</td>
<td>-25.9</td>
</tr>
</tbody>
</table>

The data indicate that conjugation of OPN-antibody onto LN and encapsulation of CP drug did not significantly increase the average size of the nanocarriers (some modest increases were observed but not statistically significant). The PDI value reflects the distribution of
nanocarrier size. PDI values <0.3 in all samples suggest that the conjugation also did not lead to the formation of some excessively large particulate matters. The modestly negative zeta potentials as shown in the table help to maintain the dispersed state of OPN-LN. In general, OPN-antibody conjugation and loading of drug do not have any detrimental impact on the size/charge aspect of the lipid nanocarriers.

c. Dispersion stability study (under Objective 1). To rule out the possibility OPN-LN forming aggregates that may affect their interaction with the target cells, their size at different conditions (4 °C or 37 °C, up to 5 days) was monitored using dynamic light scattering. The results are summarized as below:

![Graph showing size vs. storage conditions](image)

It is shown that OPN-LN remained similar in size even at 37 °C for 2 days. Some increase in size suggesting modest aggregation occurred after 5 days of incubation. In general, the OPN-LN can be considered fairly resistant to aggregation and this should not be a major factor affecting the cell-nanocarrier interaction.

d. Characteristics of OPN-targeting nanoparticles (OPN-LP) delivering GDC-0449 (i.e. Vismodegib) (Objective 1). Later, as we recognized that more specific, potent hedgehog inhibitors are available, we decided to prepare OPN-LN using the new generation drugs. GDC-0449 (brand name Vismodegib) was chosen because it represents the first hedgehog signaling pathway that has gained the approval of FDA. Comparing to cyclopamine, Vismodegib is more
potent (literature IC50 value of cyclopamine is more than 10-fold higher) and selective on sonic hedgehog pathway. The potential side effects will be lower using this newer drug. The resulting Vismodegib-OPN-LN (Vis-OPN-LN) has diameter at 118 +/- 18 nm (mean +/- SD; n=4, See Figure 1) with uniform size (polydispersity index averages 0.178 +/- 0.041). Drug encapsulation efficiency was 72.4 +/- 4.6%. The nanoformulation showed no significant size growth, aggregation and precipitation at 37 °C in 72 hours, indicating good physical stability under the conditions used for the cell culture experiments.

e. Electron microscopy (EM) (under Objective 1): To double-check the size data from dynamic light scattering and learn the morphology of OPN-LN, transmission electron microscopy imaging was performed. A representative image is shown as follows:

![Electron Microscopy Image](image)

OPN-LN was shown to be spherical and regular in morphology. The size is consistent with the dynamic light scattering measurement.

f. OPN-antibody conjugation (under Objective 1). OPN-LN was prepared with different antibody to LN ratios. ELISA was performed to evaluate the amount of unconjugated, free antibody and the result obtained was used to determine the efficiency of antibody conjugation (amount of antibody conjugated x 100%/ amount of antibody added). The result shows a conjugation efficiency of the nanoformulation at over 30%.

g. Confirmation of OPN expression in prostate cancer cells (Objective 2): To determine the OPN expression levels in different human prostate cancer cell lines, Western blotting analysis
was performed using anti-human OPN mouse IgG as primary antibody and goat anti-mouse IgG-HRP as secondary antibody.

OPN was expressed in all four prostate cancer cells tested (From left to right: PC-3M, Du145, LNCap, PC3). The strongest OPN expression was observed in PC-3M. PC-3M is a metastatic subline of PC3 prostate epithelial cancer cells. Because of its high expression it was chosen as the primary cell line for subsequent in vitro studies.

**h. Evaluate interaction of OPN-LN and OPN-expressing prostate cancer cells (Objective 2):**

To study the correlation between OPN-antibody conjugation and interaction with the OPN expressing prostate cancer cells, fluorescent microscope imaging was performed. PC-3M cells were used and LN were labeled with FITC-conjugated DSPC-PEG. Representative images are shown below (from left to right: non-targeting LN, OPN-LN with low density of OPN antibody, OPN-LN with high density of OPN antibody).

The cellular uptake of LN noticeably improved with surface decoration OPN-antibodies on the LN. This demonstrates the feasibility of using OPN-targeting for improved nanoparticle-cell interaction.

**i. Evaluate baseline cell toxicity of OPN-LN (Objective 3):** Sometimes active targeting can render a nanocarrier inherently more toxic to cells (even without drug). This may not necessarily be beneficial as the toxicity derived from the nanocarrier device itself is often less disease specific and may affect the non-target cells. We performed MTT viability assay using prostate cells with low OPN expression level (PC3). There is no significant difference between the viabilities of PC3 cells treated with OPN-LN, non-targeting LN and vehicle control (all without
any drug encapsulated). The data indicate that OPN-antibody conjugation did not affect the baseline toxicity of the nanocarrier.

2. Specific Aim 2. To study the therapeutic effects of OPN-targeted delivery on metastatic prostate cancer

a. Preparation of prostaspheres. Formation of cell spheres from individual cells indicates that these cells have tumor-initiating potential. Prostaspheres formed from individual PC-3M cells were prepared (See Figure below, left panel). Prostaspheres generally formed approximately 10-14 days after initiation, and the resulting prostaspheres were stable (no sign of spontaneous disintegration) over the course of 7 more days without drug treatment (Fig., right).

Prostasphere of PC-3M cells. Left: Microscope image of prostasphere. Cell nuclei stained for visualization. Right: Number of prostaspheres remained intact for extended culture (started with 10 prostaspheres on day 0, 3 experiments).

b. Effect on formation of prostaspheres. Successful suppression of prostasphere formation is an indication that the treatment may have potential activity to suppress renewal of tumor-initiating cells to form micrometastases. We evaluated the effect of different treatments on formation of prostaspheres from individual PC-3M cells. Treatments including free Vismodegib, blank OPN-LN and Vis-OPN-LN were compared. Cells were seeded and treated for 3 days, and then allowed to form prostaspheres. Fig. 3 shows that without treatment (at 0), around 7.5% cells eventually formed prostaspheres. Blank OPN-LN did not have significant effect except at the highest concentration tested. Vis-OPN-LN was similarly effective to free drug in suppressing the prostasphere formation at high levels (100 µM or above) and was significantly more effective at
lower concentrations. The data indicate that Vis-OPN-LN is comparable or even modestly better than the free drug in suppressing the repopulation of prostate cancer cells. It should be noted that this is an in vitro result. In in vivo data with the passive and active tumor targeting properties the local drug concentration in the tumor when delivered by Vis-OPN-LN should likely be higher than the free drug. This issue will be addressed in future in vivo studies.

![Graph showing effect of Vismodegib nanoparticles on formation of PC-3M prostaspheres.](image)

**Effect of Vismodegib nanoparticles on formation of PC-3M prostaspheres.**

c. **Effect on existing prostaspheres.** We also evaluated the effect of the treatments on existing prostaspheres. They were treated with free drug or Vis-OPN-LN for 5 days, and the prostaspheres trypsinized and MTT assay was performed. Results are normalized and expressed as % of untreated control (Fig. 4). It can be seen that the nanoparticles were significantly more effective than the free drug in killing the cells in existing prostaspheres.
d. Pharmacodynamic effect of Vis-OPN-LN on Smo, MMP-2 and MMP-9. We attempted to evaluate the change in expression of the primary target of hedgehog inhibitors, i.e. Smo and other stromal factors that may be expressed in tumor microenvironment, e.g. MMP2 and MMP9, indicating tumor aggressiveness and invasiveness. Western blotting was performed, the bands were analyzed with ImageJ software (NIH freeware) and representative result is shown as below.

Vis-OPN-LN reduced the expression of Smo and MMP-2 to 43% and 48% of the blank control, respectively, and was modestly better than free drug (12% and 6% lower than Free Vis group). The effect on MMP9 is minimal. Overall Vis-OPN-LN demonstrated noticeable pharmacodynamics effects including Smo, the primary target of hedgehog inhibitor. At a minimum, its effects are at least comparable to the free drug.

Goals to be completed in coming reporting period:

Not applicable (this is the final report)

- **What opportunities for training and professional development has the project provided?**
  Nothing to report

- **How were the results disseminated to communities of interest?**
  Nothing to report

- **What do you plan to do during the next reporting period to accomplish the goals?**
  Not applicable (this is the final report)
4. IMPACT:

- What was the impact on the development of the principal discipline(s) of the project?

  Overall, the findings in the research funded by this exploratory grant have led to successful development of a novel lipid-based nanocarrier that can deliver hedgehog inhibitors (e.g. Vismodegib) with targeting properties to osteopontin, a protein that tends to express on advanced prostate cancer cells and their microenvironment. The resulting nanoformulations Vis-OPN-LN has good interaction with metastatic prostate cancer cells such as PC-3M which express high OPN level, and can both prevent prostaspheres from forming and kill the cells in existing prostaspheres. In other words, Vis-OPN-LN has the potential to target the cancer cells with tumorigenic potential, which has strong significance in cancer treatment as this cell subpopulation are probably the ones that lead to cancer cell repopulation after chemotherapy.

  The finding in Western blotting, while demonstrating that the nanoformulations has good activity suppressing the key target (Smo) of the sonic hedgehog pathway, which plays a critical role in cancer metastasis and progression, the effects are still more modest than initially expected. The nanoformuation probably still has room to improve. As the free Vismodegib has similar (and actually a bit weaker) effects, in the future studies we may consider a more potent hedgehog inhibitor. Regardless, this study has already demonstrated the feasibility of this new OPN-targeted nanotherapeutic strategy.

- What was the impact on other disciplines?
  Nothing to report

- What was the impact on technology transfer?
  Nothing to report

- What was the impact on society beyond science and technology?
  Nothing to report
5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**
  No significant changes in the approach.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  Nothing to report

- **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

- **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:

Publications, conference papers, and presentations


- A manuscript is close to submission pending on some polishing.
7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:**

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

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<tr>
<th>Name</th>
<th>Ho-Lun Wong</th>
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<tr>
<td>Project Role:</td>
<td>PI</td>
</tr>
<tr>
<td>Researcher Identifier ORCID ID:</td>
<td>0000-0002-0349-7708</td>
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<td>Nearest person month worked:</td>
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<td>Contribution to Project:</td>
<td>He designed the project, performed nanocarrier preparation and characterization, conducted some in vitro works, and is mainly responsible for data analysis and manuscript writing</td>
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<td>Funding Support:</td>
<td>NIH 1R01CA168917</td>
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<tr>
<th>Name</th>
<th>Jan Romano</th>
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<tr>
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<td>Part time technician</td>
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<tr>
<td>Researcher Identifier (e.g. ORCID ID):</td>
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<tr>
<td>Contribution to Project:</td>
<td>Ms Romano has assisted Dr. Wong in nanoparticle preparation and characterization, and was responsible for cancer cell culture and also assisted in some in vitro experiments</td>
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<tr>
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</tr>
<tr>
<td>Researcher Identifier (e.g. ORCID ID):</td>
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<td>Contribution to Project:</td>
<td>Ms Tran followed up on the technical tasks after Ms. Romano left for a new job. She helped prepare the cell spheroids and assisted in the in vitro experiments</td>
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- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
  Nothing to report

- What other organizations were involved as partners?
  Nothing to report
8. SPECIAL REPORTING REQUIREMENTS
Nothing to report

9. APPENDICES: Nothing to report