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14. ABSTRACT
The objective of this research is to design and develop a nanocarrier that is able to evade the immune system, circulate in the blood stream, find its target prostate cancer cells, and transfer therapeutic genes into prostate cancer cells efficiently. The gene carrier is composed of: a) histone H2A peptide to condense pDNA into nano-size particles, b) a PC-3 specific targeting motif (TM) to target prostate cancer cells, c) an endosomolytic motif to disrupt endosome membrane, and d) a nuclear localization signal (NLS) to actively translocate pDNA towards the nucleus of cancer cells. The gene delivery system was synthesized in E.coli. The vector was then complexed with plasmid DNA (pDNA) to form stable nanoparticles with sizes below 100nm. The nanoparticles were used to deliver reporter genes (pEGFP) to target PC-3 prostate cancer cells and RWPE-1 normal epithelial prostate cells. The induction of immune response by the vector was studied in BALB/c immune-competent mice. The results demonstrated that the gene delivery system is able to target and efficiently transfect PC-3 cancer cells with minimum cross-reactivity with normal epithelial prostate cells. An animal protocol was prepared and approved by IACUC and DOD ACURO. The immunogenicity studies showed that the vector does not induce production IgG or IgM after repeated systemic injection.

15. SUBJECT TERMS
Nanocarriers, non-immunogenic, PC-3 prostate cancer targeting, vector development

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Introduction

A major obstacle to improving patients' survival with advanced prostate cancer is progression of the cancer to androgen-independence. Therefore, methods such as gene therapy capable of delay or stop this progression may have a significant impact on improving patients' health. However, many challenges lie ahead for gene therapy, including improving DNA transfer efficiency to cancer cells, enhancing levels of gene expression, and overcoming immune responses. **The overall objective** of this research is to design and develop a nanocarrier that is able to evade the immune system, circulate in the blood stream, find its target prostate cancer cells, and transfer therapeutic genes into prostate cancer cells efficiently. The gene carrier is composed of: a) histone H2A peptide (H2A) to condense plasmid DNA (pDNA) into nano-size particles (nanocarriers), b) a PC-3 specific targeting motif (TM) to target prostate cancer cells, c) an endosome disrupting motif (EDM) to disrupt endosome membranes and facilitate escape of the cargo into the cytosol, and d) a nuclear localization signal (NLS) to actively translocate pDNA towards the nucleus of cancer cells. An elastin like polymer (ELP) has also been engineered in the vector structure to provide a hydrophilic shield and protect the vector/pDNA complex in the blood stream from the immune system. For simplicity, the vector without ELP will be shown as EDM-H2A-NLS- TM or namely GHT2 and the vector equipped with ELP will be shown as GHET2. PC-3 prostate cancer cells are selected as target because they are highly metastatic and characterized to be $CAR^{-}/HER2^{-}$. This means that they are not a good candidate for adenoviral gene therapy or Herceptin anti-HER2 immunotherapy. Therefore, development of a targeted delivery system for this type of prostate cancer cells could be highly beneficial.

CAR: Coxsackie Adenovirus Receptor

HER2: Human Epidermal Growth Factor Receptor 2

Body

This is the third year report; therefore, below we have highlighted the studies that have been performed in Year 3 of the DOD grant. We have met all the objectives of Years 1 and 2 and our annual report for years 1 and 2 have been approved by DOD reviewers. Because the PI changed institutions, some of the deadlines for year 3 as delineated in the Statement of Work are not met in time. As a result, we requested a one year no-cost extension which was approved by the DOD. The following three tasks were proposed for year 3:

Task 1- Inject the nanocarriers in mice bearing xenograft tumor model of prostate cancer and evaluate the transfection efficiency and therapeutic efficacy (months 1-11). This task will be performed in the Department of Pharmaceutics, rooms 202-206 and vivarium at Rutgers University by the PI and the Postdoctoral Fellow in collaboration with Dr. Tamara Minko.

1.1. To evaluate transfection efficiency, pCMV-luc or pEGFP will be complexed with vectors and used to transfect PC-3 tumors in nude mice. The transfection efficiency in tumors and other tissues will be evaluated using an in vivo imaging system. These studies will be performed using 40 nude mice.

- The animal protocol for this task was prepared and approved by the IACUC (Protocol #: 10-070). The approval from DOD Animal Care and Use Review Office (ACURO) has also been obtained. However, we have not completed the subtasks 1.1 and 1.2 yet because the PI changed institution and it took some time to hire new personnel and train them to perform the proposed studies. At present we have implanted the tumors in nude mice and waiting for them to become palpable. We expect to finish subtasks 1.1 and 1.2 in 4 to 8 months.

1.2. To evaluate therapeutic efficacy, Xenograft tumors of prostate cancer will be established in nude mice. The mice will be treated with vector/pSR39 complexes and controls. The size of tumors will be measured at various time points. Apoptotic activity of SR39 in tumor tissue versus normal tissues will be studied by TUNEL apoptotic assay. These studies will be performed using 48 nude mice.

- This task is in progress.

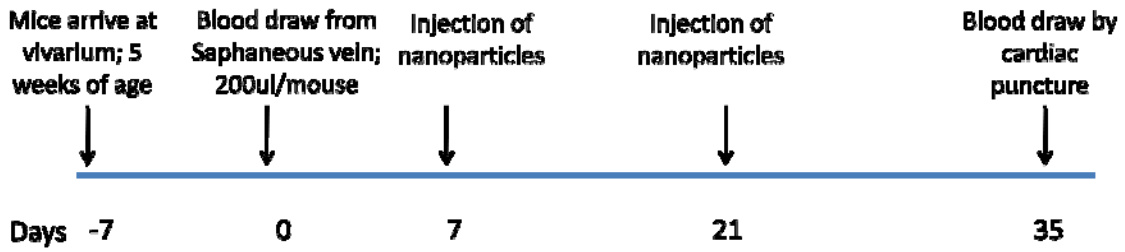
Task 2- Evaluate immune system response to the nanocarriers using immune-competent mice (months 9-12). This task will be performed in the Department of Pharmaceutics Rooms 202-206 and vivarium at Rutgers University by the PI and the Postdoctoral Fellow in collaboration with Dr. Minko.

2.1. To test the immunogenicity of the vector, the production of anti-vector antibodies will be evaluated in mice. Intact male BALB/c mice will be given an intravenous injection of vector/pDNA nanocarriers or controls on days 7 and 21 after the initial challenge, for a total of two injections. The anti-vector antibodies will be assessed by ELISA. This study will be performed using immunocompetent mice (months 9-12).

- Male Balb/c mice (5-6 weeks old; Jackson laboratories; Maine, USA) were immunized via tail vein (i.v.) with the following formulations:

1- pCpG free plasmid only (1μg)
2- pBudce4.1 only (1μg plasmid DNA with CpG island)
3- Polyethyleneimine in complex with 1μg pBudce4.1
4- PEGylated liposomes
5- GHT2 in complex with 1μg pBudce4.1
6- GHET2 in complex with 1μg pBudce4.1

Nanoparticles were injected on Day 7 and Day 21. This dosing schedule was in accordance with previous studies conducted by Mumper et al [2]. The study design is illustrated in the scheme below:



Briefly, Balb/c mice were housed for one week after arrival for acclamation (Day minus7). On Day zero, blood was collected after shaving the hair off by saphaneous vein puncture from both legs using a 3mm lancet (GoldenRod). Approximately 200ul of blood was collected in heparinized micro-Hematocrit tubes (Kontes). Blood from the capillary tubes were transferred to EDTA-coated microtainer tubes (BD). Plasma was collected by centrifugation of samples at 4000rpm for 20 minutes and stored at -80^{0}C until further evaluation. On day 7and day 21, animals were immunized via tail vein using a 27G needle with the nanoparticle formulations and controls as mentioned above. On day 35, animals were euthanized using CO_2 and blood drawn via cardiac puncture using a 25G needle. Blood was collected into EDTA-coated microtainer tubes (BD). Plasma was collected by centrifugation of samples at 4000rpm for 20 minutes and stored at -80^{0}C until further evaluation.

ELISA was performed to determine IgG and IgM levels as per manufacturer's instructions using the IgG and IgM ELISA kits from Bethyl laboratories.

The results of the experiments showed that neither GHET2 nor GHT2 induced production of IgG and IgM after repeated injections (Fig 1).

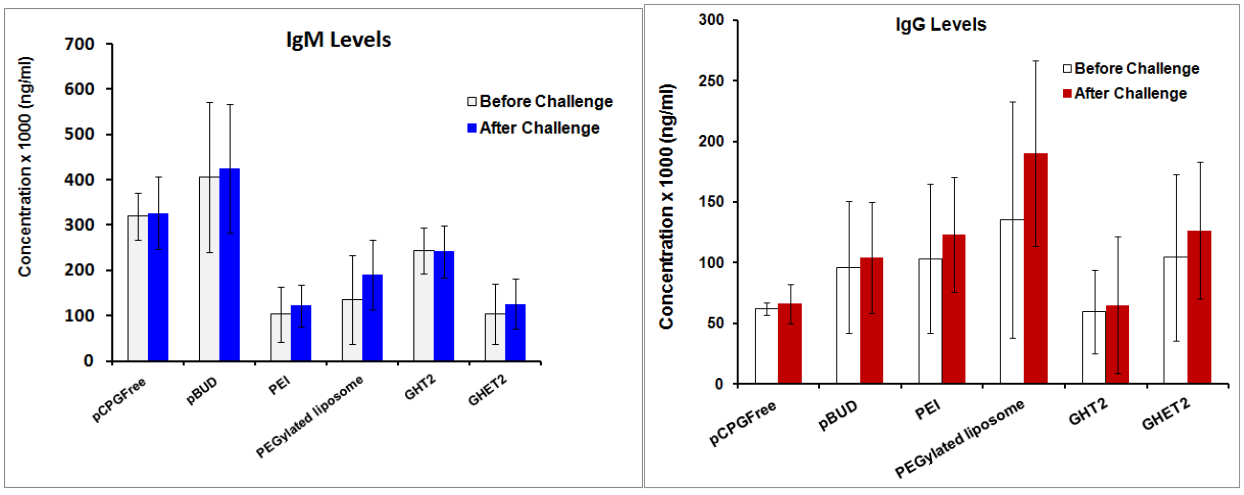


Fig. 1: Elisa studies for the evaluation of IgG and IgM production against antigens after repeated injections in immune-competent mice.

Task 4: Writing manuscripts, final report, and dissemination of the data in a conference (**Months 11-12**).

- We are in the process of preparing a comprehensive manuscript that includes the results of all the proposed studies. This manuscript will be submitted for publication as soon as the results of the proposed animal studies are obtained.

Key Research Accomplishments

a) Developed stable nanoparticles with sizes less than 100nm. This size range makes them suitable for receptor-mediated endocytosis.
b) Formulated nanoparticles with ability to target PC-3 prostate cancer cells with high efficiency but with low binding to normal epithelial prostate cells.
c) Preliminary analyses of data obtained from the immunogenicity studies shows that our vectors are not immunogenic.

Reportable Outcome

A) Manuscript: In preparation

B) Presentations:

1- The PI (A. Hatefi) submitted an abstract and presented this work in Cancer Institute of New Jersey Annual Meeting (2012) held in Piscataway, NJ.

C) Training: A Research Scientist with experience in molecular biology techniques was hired. She has received training in vector development, vector characterization, mammalian cell culture and transfection, and targeted prostate cancer gene therapy. In addition, a PhD student was trained to perform the studies. The newly recruited PhD student will be conducting the proposed animal studies under Task 1.

D) Grant application: None

E) Patent application: Depending on the outcome of the therapeutic efficacy studies, there is a possibility that we file a provisional patent.

Conclusions

Using genetic engineering techniques we have created a PC-3 specific gene delivery system that does not induce production of IgG and IgM and can *potentially* be used in the treatment of the patients that do not respond to adenoviral gene therapy or Herceptin immunotherapy.

References

[1]- Y. Wang, S.S. Mangipudi, B.F. Canine, **A. Hatefi**, A designer biomimetic vector with a chimeric architecture for targeted gene transfer. *J Control Release* 137 **(2009)** 46-53.
PMID: 19303038

[2]- Anekant Jain, Weili Yan, Keith R. Millerc, Ronan O'Carrad, Jerold G. Woodwarde, Russell J. Mumper. Tresyl-based conjugation of protein antigen to lipid nanoparticles increases antigen immunogenicity. *Int J Pharm* 401 (2010) 87–92)

Appendices

Appendix A: The published abstract for Cancer Institute of New Jersey Annual Meeting (2012).

A Recombinant Non-Viral Vector for PC-3 Prostate Cancer Gene Therapy

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Objectives: The objective of this research was to develop a multifunctional vector that can condense therapeutic genes into nano-size carriers, target PC-3 prostate cancer cells and transfect them efficiently. To achieve the objective, we developed a recombinant vector composed of four repeating units of histone H2A, a fusogenic peptide and a PC-3 targeting motif. The PC-3 cancer cell model was chosen as a target because it represents a subpopulation of aggressive prostate cancer cells with potential for metastasis. In addition, PC-3 cells don't express coxsackie adenovirus receptors which make them a poor candidate for adenoviral gene therapy.

Methodologies: The vector was designed, genetically engineered in E. coli and purified using nickel column chromatography. It was complexed with pEGFP (encodes GFP) to form nanosize particles. The size of the nanoparticles was measured using dynamic light scattering technique. Vector/pEGFP complexes were used to transfect PC-3 (prostate cancer) and RWPE-1 (normal prostate) cells. The transfection efficiency was determined using flow cytometry. The vector-related cytotoxicity was also determined by a WST-1 cell toxicity assay.

Results: The results demonstrated that the vector is able to condense plasmid DNA into particles with sizes less than 100 nm, target PC-3 cancer cells but not RWPE-1 and mediate efficient gene expression. The cell toxicity assay data also showed that the vector by itself does not have any detectable toxicity at its maximum efficiency.

Conclusions: Recombinant DNA technology allowed us to create a targeted multifunctional vector that can specifically transfect PC-3 prostate cancer cells with minimal impact on normal prostate cells. Using cancer suicide genes, the in vivo therapeutic efficacy studies are in progress.

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