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Prostate Cancer

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Salt Lake City, Utah 84102

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*Biosketches*

Jerald C. Hinshaw

Jiang Sha

## A. Introduction

In this project, we are developing a new approach toward stimulating a patient's own immune system as a therapeutic approach for the treatment of cancer in general, and prostate cancer, in particular. Thus far, immunotherapeutic approaches for treating cancer (including prostate cancer) in the clinical setting have not been uniformly successful<sup>1</sup>. Many of these methods have been directed toward the stimulation of a CD8+ T-cell response in the host, but have lacked a mechanism for vigorous immune system stimulation and have not combined CD4+ T-cell responses or B-cell involvement<sup>2</sup>. Recently, techniques involving stimulation of dendritic cells (DC) with tumor antigens have begun to show promise, but the methods involve isolation of DCs and *in vitro* stimulation, followed by re-injection into the patient. Our methodology involves direct *in vivo* stimulation/maturation of DCs *via* Toll-like receptors (TLRs)<sup>3-5</sup>. We are chemically synthesizing molecular conjugates that comprise a TLR ligand covalently linked to a prostate cancer tumor-associated antigen protein or peptide. Using this tactic, we anticipate that the TLR-ligand portion of the conjugates will stimulate DCs (as well as other TLR-expressing antigen presenting cells) to secrete immune-activating cytokines while the tumor antigen component of the complex will be processed and presented to activated T cells. We have addressed the synthesis of conjugates based on TLR-2 and TLR-7 ligands and will soon examine a TLR-4 ligand<sup>6,7</sup>. The prostate cancer antigen portion of the complexes currently includes an MHC Class I peptide epitope from prostate-specific membrane antigen (PSMA)<sup>8</sup> and a general MHC Class II peptide epitope (PADRE)<sup>9,10</sup>. Soon, we expect to include the complete prostate-specific antigen (PSA) protein linked to each TLR ligand. Our conjugates are water-soluble and represent a new and potent immune system stimulation and antigen presentation mechanism with *in vivo* activation of DCs aimed at the stimulation of combined CD8+ and CD4+ T-cell responses along with B-cell activation through the innate/adaptive immune response connection. The conjugates are designed to elicit a vigorous immune response to prostate cancer and may be administered by sc injection, nasal, or possibly oral routes.

## B. Body

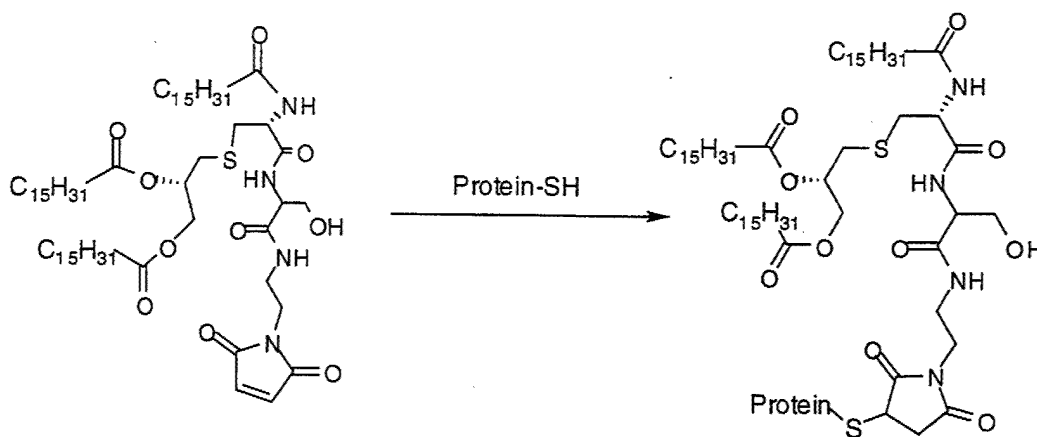
This section describes research accomplishments associated with the tasks outlined in the original award application thus far.

### Task 1. Synthesize lipopeptide carriers covalently conjugated to prostate-specific antigen (PSA) protein (months 1-14)

We are preparing (with first year graduate student Jiang Sha) the PSA protein, which contains a carboxyl terminal cyteine residue for chemical attachment to our TLR ligands. The *Pichia pastoris* yeast expression system employed previously to express the native PSA protein, is being utilized<sup>11</sup>. Primers that contain a carboxyl terminal cysteine (5NPSA: 5'-cccttctcgagaaaagaattgtggga-3', PSASac: 5'-taaccgcggttagcaggggttgcca-3') were designed and PCR was performed with the plasmid proPSA/pET-12 (kindly provided by Dr. T. Takayama, University of Washington) as the template. The primers were designed such that digestion of the PCR product and the *Saccharomyces cerevisiae* expression vector pPICZalphaA using restriction enzymes XhoI and Sac resulted in an inframe

fusion of the mature PSA (no propeptide) sequence with the yeast  $\alpha$ -factor signal sequence. Restriction enzyme digestion of the vector and insert was done separately. Ligation was performed at room temperature for two hours. The ligation mixture was transformed into *E. coli* strain TOP10. After culture on low salt Luria broth agar plates that contained 25  $\mu\text{g/ml}$  Zeocin, Zeocin-resistant colonies were obtained. From eight colonies, one plasmid with the correct sequence was chosen for transformation into *Pichia pastoris* strain X33. Sufficient plasmid DNA was generated and linearized in the 5'AOX1 region with the restriction enzyme PmeI for integration into the 5'AOXI region of the chromosome of *Pichia pastoris*. Because pPICZalphaA does not contain a yeast origin of replication, transformants can only be isolated if recombination occurs between the plasmid and the *Pichia* genome. Chemical transformation was accomplished using the linearized plasmid and competent *Pichia* cells. After a two-day incubation at 30  $^{\circ}\text{C}$ , about 50 colonies were produced. A single colony was picked and incubated in 25 ml of buffered glycerol-complex media until the cells were in log-phase growth. The cells were harvested by centrifugation and resuspended in a buffered methanol complex media for induction of the AOX1 promoter. Methanol was added to a final concentration of 0.5% in the media every 24 hours to maintain expression. After 72 hours, the supernatant was harvested by centrifugation. Unfortunately, we have been unable to detect the desired recombinant PSA (rPSA) protein (~ 28 kDa) by either reducing SDS PAGE or Western blot using mouse monoclonal anti-PSA antibody. We are currently analyzing the recombinants for the correct product.

In parallel with the rPSA effort, we have been working on the synthesis of the TLR-ligand portion of our proposed conjugates. Our initial efforts were directed toward the preparation of the TLR-2 ligand Pam<sub>3</sub>Cys (*N*-palmitoyl-bis-palmitoyloxy-(2*R,S*)-propylcysteine)<sup>12</sup> incorporating a thiol-reactive maleimide<sup>13</sup> functionality for attachment of our rPSA protein through the terminal cysteine functionality (**Scheme 1**).

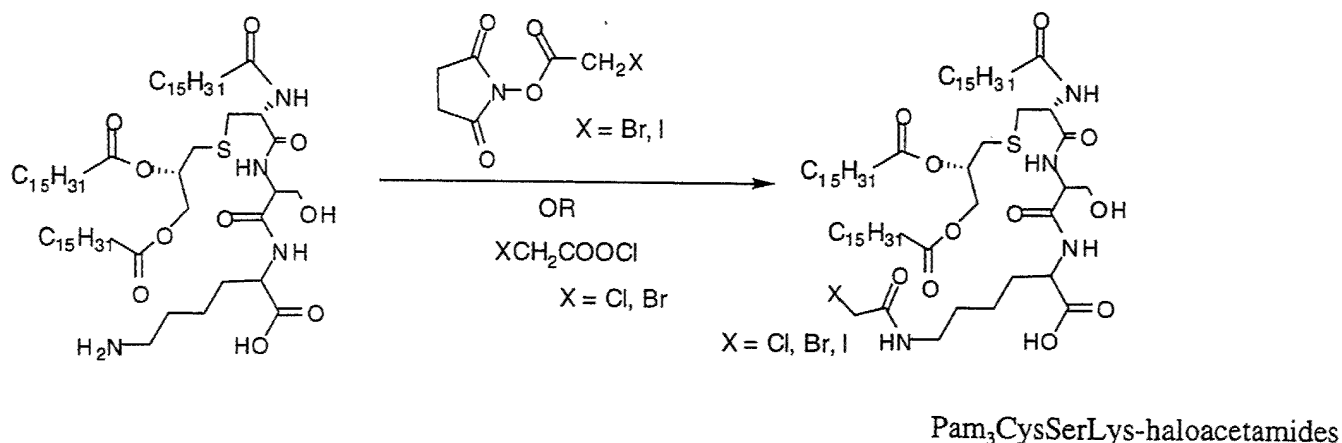


Pam<sub>3</sub>Cys-Ser-maleimide derivative

**Scheme 1**

After considerable effort, using a number of maleimide reagents as well as different synthetic procedures to incorporate a maleimide group, we have been unable to obtain a Pam<sub>3</sub>Cys-maleimide intermediate in sufficient yield or purity to be utilized.

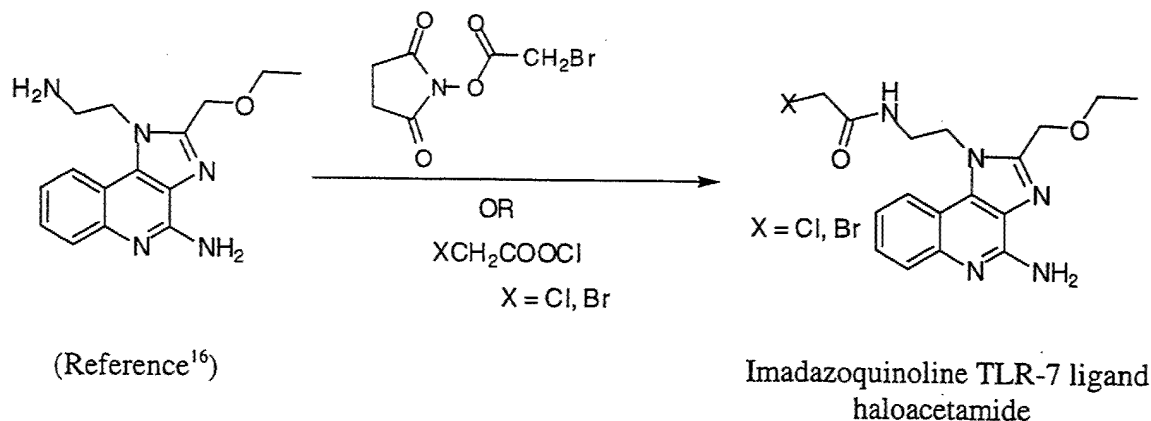
We have turned our attention to the synthesis of Pam<sub>3</sub>Cys derivatives incorporating the thiol-reactive haloacetamide functionality (**Scheme 2**). Bromo, chloro, and iodo



**Scheme 2**

Pam<sub>3</sub>Cys derivatives have been prepared. It presently appears that the iodo derivative is too reactive for use in our synthetic scheme. We are currently evaluating the reactivity of the chloro and bromo derivatives with thiol-containing peptides to decide upon the most suitable intermediate for our application.

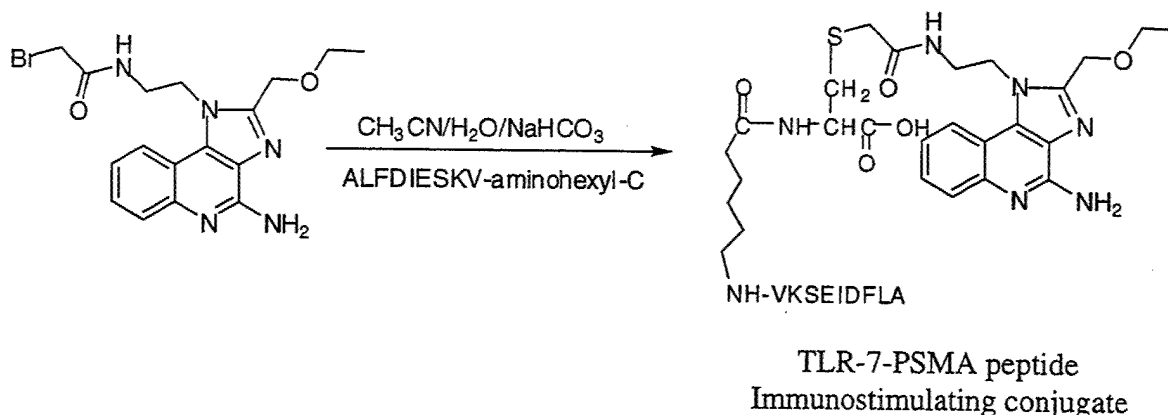
Concurrently with the Pam<sub>3</sub>Cys work, we have prepared a TLR-7 ligand derivatized with chloro and bromo haloacetamide functionality (**Scheme 3**). The ligand is based on the imidazoquinoline<sup>14,15</sup> class of immunostimulants in which the mode of action has recently been



(Reference<sup>16</sup>)

**Scheme 3**

identified as activation of the TLR-7 receptor<sup>17</sup>. Using the TLR-7 haloacetamide intermediates, we have prepared an immunostimulatory conjugate based upon the PSMA HLA-A2 restricted peptide epitope (ALFDIESKV)<sup>8</sup>, which incorporates an aminohexyl spacer between the peptide epitope and a terminal cysteine for attachment to the TLR ligands (**Scheme 4**).



**Scheme 4**

We are currently preparing the TLR-7 conjugate with the general CD4+ HLA-DR restricted peptide epitope PADRE<sup>9,10</sup> linking the same aminohexyl functionality to a terminal cysteine (aK-clohexylalanine-VAAWTLKAAa-aminohexyl-C)<sup>9,10</sup>. Soon we will prepare the analogous Pam<sub>3</sub>Cys conjugates with the PSMA peptide and PADRE. With the availability of rPSA, we will also be in a position to prepare the analogous TLR-2 and TLR-7 immunostimulating complexes with the complete PSA protein.

**Task 2.** Immunize HLA-DR4 and HLA-2A transgenic mice with lipopeptide-PSA conjugates. Analyze immune responses and test responses against prostate cancer cell-lines (months 9-25)

With the availability of synthesized immunostimulating conjugates we are on schedule and are now preparing for mouse immunization experiments. Transgenic mice have been ordered, and the initial immunization experimental plans are being finalized. Prostate cancer cells (LNCaP) have been obtained and cultures are being established.

**Task 3.** Prepare a PSMA covalent conjugate having a superior lipopeptide carrier determined from **Task 2**. Perform mouse immunization experiments. Test responses against prostate cancer cell-lines (months 20-36)

This task is scheduled for later in the program.

### **C. Key Research Accomplishments**

This section provides a list of key accomplishments for the first year from this research.

- Ligands recognized by TLR-2 and TLR-7 have been prepared.
- These ligands were chemically modified with thiol-reactive haloacetamide functionality for attachment of cysteine-containing antigenic proteins and peptides.
- TLR-7 ligand-antigenic peptide conjugates have been prepared.
- All newly-synthesized compounds have been purified and chemically characterized.
- Plasmids to be used for expressing rPSA protein with a terminal cysteine for chemically linking to TLR ligands have been designed and expressed.

### **D. Reportable Outcomes**

Graduate research assistant, Mr. Jiang Sha, is supported by this program, and the results from his research will be incorporated into his dissertation.

### **E. Conclusions**

Research on this program thus far has provided modified TLR-2 and TLR-7 ligands suitable for chemical attachment of prostate cancer associated antigenic peptides and proteins. The resulting immunostimulating conjugates are ready for use in mouse immunization experiments in order to ascertain the production of CD8+ and CD4+ T cells as well as antibodies specific to prostate cancer antigens.

This research is significant in that it represents the first attempt to generate an immune response to prostate cancer antigens from the potent immune system activation that arises from the innate/adaptive response connection utilizing targeted TLR signaling in combination with antigen presentation. Evaluation of the separate conjugates will provide new insight into the effectiveness of the immune response to the same antigen stimulated by different TLRs. Such effects are not yet known. Furthermore the possibility of an increased immune response to cancer antigens presented simultaneously with more than one TLR receptor activation path has not been explored. With our newly-synthesized conjugates, we are in a position to initiate these exciting studies.



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**G. Appendix**

*Biosketches*

Jerald C. Hinshaw, Principal Investigator

Jiang Sha, Graduate Research Assistant

### BIOGRAPHICAL SKETCH

Provide the following information for the Principal or Co-Principal Investigators  
Follow this format for each person.

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INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Oregon State University, Corvallis, Oregon	BS	1962 - 1966	Chemistry
The University of Utah, Salt Lake City, Utah	PhD	1966 - 1970	Organic Chemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

#### Research and Professional Experience

- 1970-1978** Advanced from Senior Research Chemist to Research Associate, Organic Research Laboratory, Chemistry Division, Research Laboratories, Eastman Kodak Company
- 1978-1984** Scientist, Research and Development Laboratories, Thiokol Corporation
- 1980, 1986** Member, Utah Award Committee, Salt Lake Section, American Chemical Society
- 1981** Visiting Research Associate, University of Utah.
- 1981-1983** Chairman-Elect, Chairman, Past-Chairman, Salt Lake Section, American Chemical Society
- 1984-1990** Supervisor, Propellant Research Section, Research and Development Laboratories, Thiokol Corporation
- 1990-1999** Manager, Energetic Materials Research Department, Research and Development Laboratories, Thiokol Propulsion, Brigham City, Utah.
- 1996-1999** Member, State Advisory Council on Science and Technology (State of Utah, Governor appointment)
- 1997, 1998** Member, Utah State Governor's Medal for Excellence in Science and Technology Award Committee
- 1997-1999** Chairman, State Advisory Council on Science and Technology (State of Utah, Governor appointment)
- 1997-1999** Member, Utah Centers of Excellence Program Advisory Council (State of Utah, Governor appointment)
- 2/99-7/99** Senior Staff to the Technical Director, Science and Engineering, Thiokol Propulsion, Brigham City, Utah
- 7/99-11/01** Research Assistant Professor, Department of Medicinal Chemistry, The University of Utah, Salt Lake City, Utah
- 11/01-current** Research Associate Professor, Department of Medicinal Chemistry, The University of Utah, Salt Lake City, Utah

## Research Interests:

Synthetic chemistry  
Synthesis of bacterial oxidosqualene cyclase inhibitors  
Cancer immunotherapy  
Targeted drugs  
Design and synthesis of small molecule inhibitors of protein-protein signaling  
Design and synthesis of fluorescent phosphoinositide probes  
Research and technology management.

## Honors:

Listed in "American Men and Women of Science"  
Listed in "Who's Who in Technology"  
Named Outstanding Senior in Chemistry, 1966  
National Defense Education Act Title IV Fellow, 1968-1970  
Franklin Award, Thiokol Corporation recognition for outstanding technical achievement, 1995

**Publications/Patents:** J. C. Hinshaw has over 50 publications and patents. Those for 2000-2003 are listed.

A. Ponstler, A. Silva, A. St. Hilarie, L. Tjoelker, Y. Xu, J. Hinshaw, G. Prestwich, G. Zimmerman, and T. McIntyre "Lysophosphatidic Acid is a transcellular PPAR $\gamma$  Agonist", *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 131-136.

S. Davies, A. Pontsler, G. Marathe, K. Harrison, R. Murphy, J. C. Hinshaw, G. D. Prestwich, A. Hilaire, S. Prescott, G. Zimmerman, and T. McIntyre, "Oxidized Alkyl Phospholipids are Specific, High Affinity PPAR $\gamma$  Ligands", *J. Biol. Chem.*, 2001, **276**, 16015.

J. C. Hinshaw and G. D. Prestwich, "The Design, Synthesis, and Evaluation of Molecules That Enable or Enhance Cellular Uptake: Peptoid Molecular Transporters", *ChemTracts, Organic Chemistry*, 2001, **14**, 391. Commentary on the research by P. Wender, D. Mitchell, K. Pattabiraman, E. Pelkey, L. Steinman, and J. Rothbard, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 13003.

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R.B. Wardle, R.M. Hajik, J.C. Hinshaw, T.K. Highsmith, "Process for the Large-Scale Synthesis of 4,10-Dinitro-2,6,8,12-Tetraoxa-4,10-Diazatetracyclo[5.5.0.0<sup>5,9</sup>.0<sup>3,11</sup>]dodecane," U.S. Patent 6,107,483, issued August 22, 2000.

J. C. Hinshaw, D. W. Doll, R. J. Blau, G. K. Lund, "Metal Complexes for Use as Gas Generants," U.S. Patent 6,039,820, issued March 21, 2000.

G. D. Prestwich, F. S. Buckner, J. C. Hinshaw, "Methods Related to Steroid Metabolism of Parasites and Mycobacteria, and Treatment of Parasite and Mycobacterial Infections with an Oxidosqualene Cyclase Inhibitor", U.S. Patent Application filed June 16, 2000.

### BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.

NAME		POSITION TITLE	
SHA, JIANG		Graduate Research Assistant	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Peking University, Beijing, China	B.S.	1997-2001	Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

#### Research and Professional Experience:

- 2000-2001      Institution of Biophysics, Chinese Academy of Science, Bachelors' degree research
- 2001-2002      Molecular Biology Program, The University of Utah, Laboratory Rotation
- 2002-current    Graduate Research Assistant, Department of Medicinal Chemistry,  
The University of Utah, Salt Lake City