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Fort Detrick, Maryland 21702-5012

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13. ABSTRACT (Maximum 200 Words) The prospects of stimulating a patient's own immune system as a therapeutic approach to the treatment of cancer in general, and prostate cancer in particular, is intriguing. However, thus far immunotherapeutic approaches to the treatment of cancer (including prostate cancer) in the clinical setting have not been uniformly successful. We are chemically synthesizing molecular conjugates that comprise a Toll-Like Receptor (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 dendritic cells (DCs) (as well as other TLR-expressing antigen presenting cells) to secrete immune-activating cytokines. Concomitantly the tumor antigen component of the complex will be processed and presented to activated T cells. In this way, a new and potent immune system stimulation and antigen presentation mechanism aimed at the stimulation of combined CD8+ and CD4+ T-cell responses, along with B-cell activation <i>via</i> the innate/adaptive immune response connection, is being developed. We have prepared TLR-4 and TLR-7 ligands and are preparing mouse immunization experiments to test the efficacy of this new approach to cancer immunotherapy.			
14. SUBJECT TERMS prostate cancer immunotherapy, Toll-like receptors, chemical synthesis		15. NUMBER OF PAGES 14	
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A. Introduction

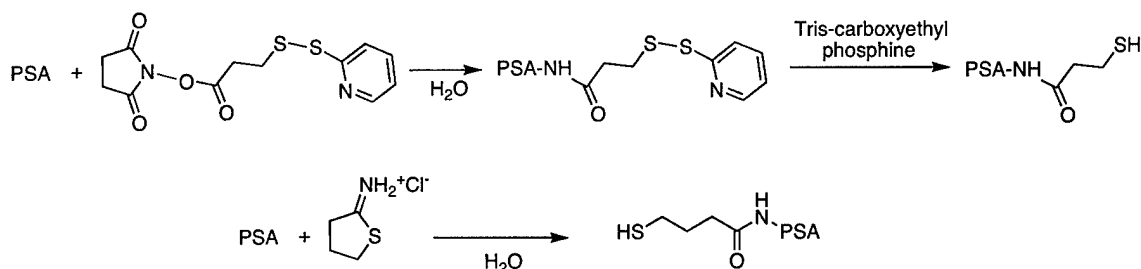
In this project, we are developing a new technique for stimulating a patient's own immune system as a therapeutic approach for the treatment of cancer in general, and prostate cancer, in particular. Thus far, immunotherapeutic protocols for treating cancer (including prostate cancer) in the clinical setting have not been uniformly successful¹. 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 involvement². Recently, techniques involving stimulation of dendritic cells (DC) with tumor antigens have begun to show promise, but the process involves isolation of DCs and *in vitro* stimulation, followed by re-injection into the patient. Our methodology uses direct *in vivo* stimulation/maturation of DCs *via* Toll-like receptors (TLRs)³⁻⁵. 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. The prostate cancer antigen portion of the complexes currently includes an MHC Class I peptide epitope from prostate-specific membrane antigen (PSMA)⁶ and a general MHC Class II peptide epitope (PADRE)^{7,8}. We have included the complete prostate-specific antigen (PSA) protein linked to a TLR-7 ligand. Our conjugates 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 s.c. injection, or possibly, nasal or oral routes.

B. Body

This section describes research accomplishments associated with the tasks outlined in the original award application for Year Two of the project.

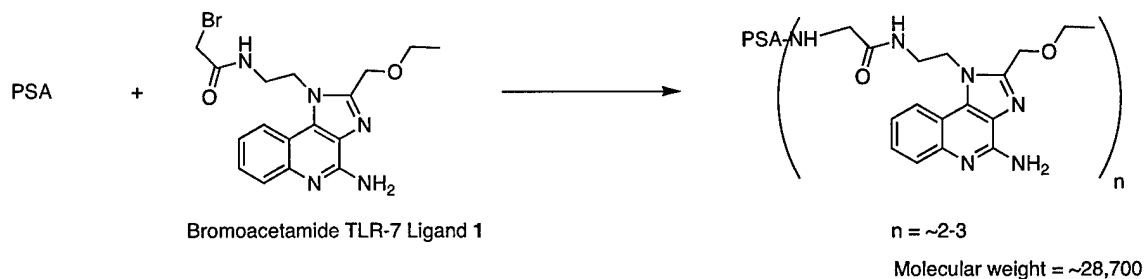
Task 1. Synthesize lipopeptide carriers covalently conjugated to PSA protein (months 1-14)

Following earlier efforts, considerable additional work was carried out during the second year of this program (with graduate student Jiang Sha) attempting to prepare the recombinant PSA protein, which contains a carboxyl terminal cyteine residue for chemical attachment to our TLR ligands. We have set aside this unproductive recombinant approach. Instead we set out to derivatize the wild-type protein. The thought was to convert one or more of the eleven PSA lysine amino groups to a desired mercaptan functionality. Human PSA was obtained from Research Diagnostics, Flanders, NJ. Several experiments aimed at chemically modifying the protein with thiolation reagents were carried out (**Scheme 1**).



Scheme 1. Attempted thiolation of PSA protein

Clean functionalization was not obtained. We suspect that the thiol-derivatized PSA may be undergoing facile intramolecular disulfide crosslinking resulting in very high molecular weight protein mixtures. Attempts to reduce these suspected disulfide linkages with dithiothreitol (DTT) or triscarboxyethylphosphine were not promising, perhaps because of unwanted concomitant reduction of one or more of the five native disulfide crosslinks in the PSA protein. These reagents are also problematic, as they would be expected to react with our bromoacetamide TLR-ligand reagents, which would require the careful removal of all traces of the reagents while maintaining the thiolated protein in the reduced state. We therefore turned to directly functionalizing the PSA lysine amino groups with bromoacetamide TLR-7 ligand **1**, which was prepared last year (**Scheme 2**).



Scheme 2. Preparation of a TLR-7- ligand- PSA conjugate

The major product(s) isolated from this reaction by HPLC was identified by MALDI mass spectroscopy as PSA appended with 2-3 TLR-7 ligands. Although the product is a mixture of functionalized proteins, we are satisfied with this material for initial immunization experiments. Using the bromoacetamide TLR ligands (**Figure 1**) that we prepared last year,

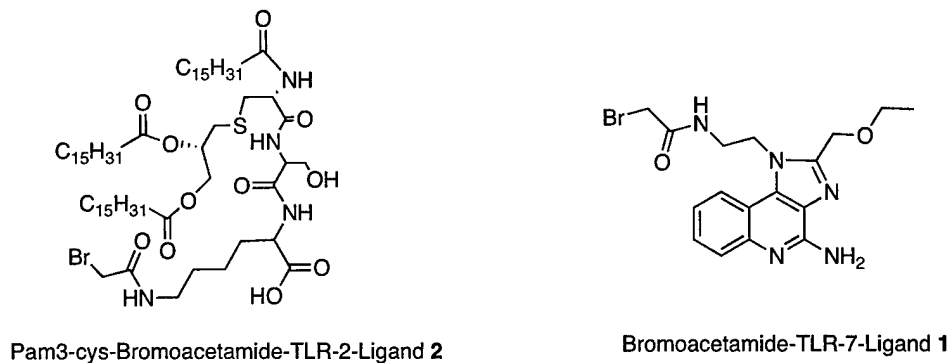
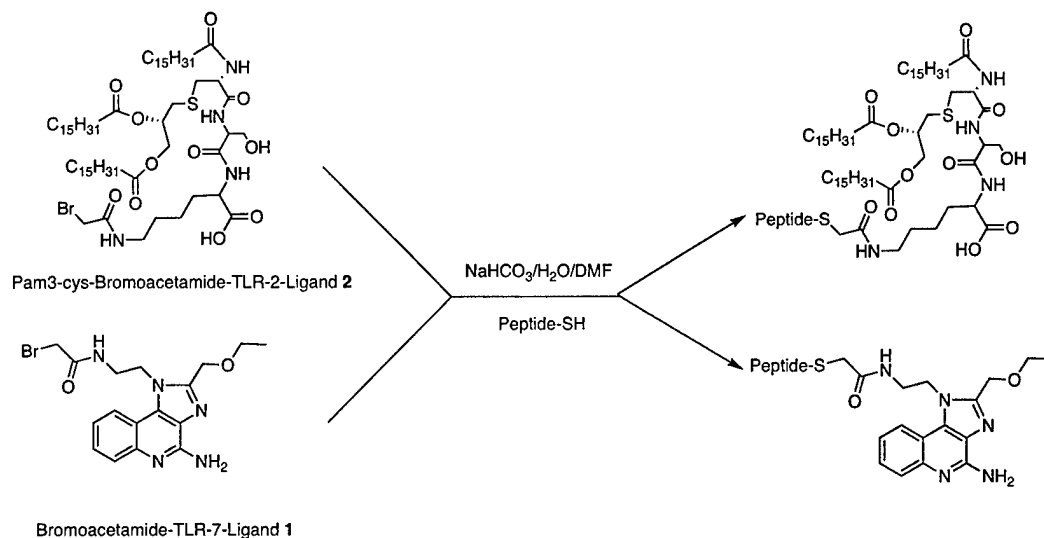


Figure 1. Functionalized TLR ligands

we have also synthesized several ligand-peptide conjugates for mouse immunization experiments. **Scheme 3** illustrates the general synthetic route used.



Scheme 3. Preparation of peptide conjugates

Figure 2 lists the compounds that have now been prepared using Scheme 3.

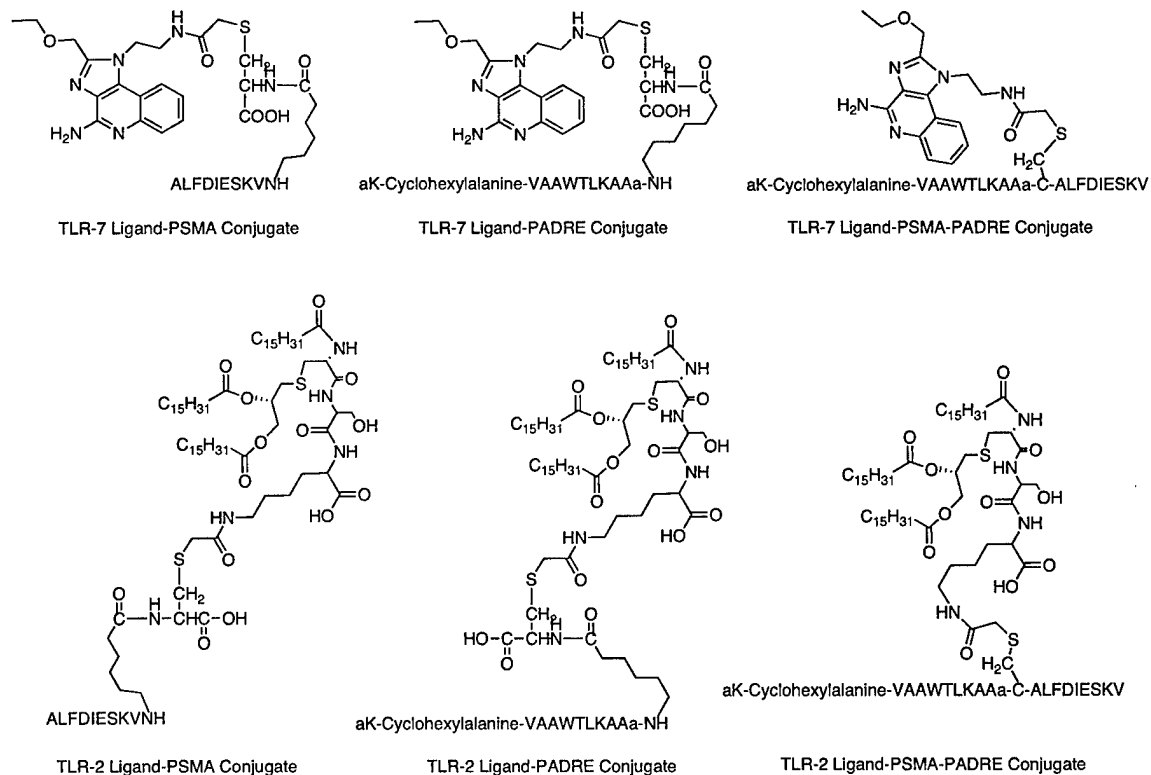


Figure 2. TLR-2 and TLR-7 ligand peptide conjugates

The PSMA immunostimulatory conjugates are based upon the human PSMA HLA-A2 restricted peptide epitope (ALFDIESKV)⁶ and incorporate an aminohexyl spacer between the peptide epitope and a terminal cysteine for attachment to the TLR ligands. The PADRE conjugates comprise the general CD4+ restricted peptide epitope PADRE^{7,8} using the identical aminohexyl functionality bonded to a terminal cyteine (aK-clohexylalanine-VAAWTLKAAa-aminohexyl-C). We also synthesized the PSMA epitope covalently linked to the PADRE peptide through a central cysteine (aK-cyclohexylalanine-VAAWTLKAAa-C-ALFDIESKV) in order to evaluate the immunogenicity of conjugates, which contain both MHC Class I and MHC Class II epitopes. The TLR-2 ligand (PAM₃-cys) lipopeptide conjugates proved to be quite insoluble, and thus far we have been unable to either adequately purify the materials or obtain mass spectral characterization data using MALDI, ESI or FAB ionization techniques. Similar difficulties have been noted recently for other PAM₃-cys-peptide conjugates⁹. For this reason, we are using the TLR-7-ligand conjugates of Figure 2 in our immunization experiments.

Task 2 Immunize HLA-DR4 and HLA-A2 transgenic mice with lipopeptide-PSA conjugates. Analyze immune responses and test responses against prostate cancer cell-lines (**months 9-25**)

Having prepared immunostimulating conjugates, we have begun mouse immunization experiments. A transgenic mouse colony has been established. Prostate cancer cell (LNCaP) cultures have been produced.

Founder homozygous mice carrying the HLA-A2.1 Enge transgene that express the human class MHC Class I antigen HLA-A2.1 on cells from the spleen, bone marrow and thymus were obtained from Jackson laboratories, Bar Harbor, ME (Stock Number: 003475). This surface protein recognizes the PSMA peptide epitope that is incorporated into our conjugates. A breeding colony of these mice has been established at The University of Utah. Since we have included the generalized human and mouse MHC Class II antigenic peptide epitope PADRE in our conjugate evaluation, it was not necessary to utilize transgenic mice expressing human HLA-DR4 as originally proposed. The PADRE peptide is expected to provide the desired CD4+ helper T-cell stimulation. Mice in groups of three were immunized (s.c., axilla) with the TLR-7 ligand PSMA-PADRE conjugate (Figure 2). Three levels of conjugate were examined (0.1, 1.0, and 10 µg). Mice were also immunized with the unconjugated PSMA, PADRE, and PSMA-PADRE peptides. Syngenic C57BL/6J mice that do not express the HLA-A2 gene were used as controls. Twenty-eight days after the initial immunization, each group of mice received a boost vaccination. Eleven days post-boost, splenocytes and peripheral lymph node (PLN) lymphocytes were harvested and [³H]-thymidine cell proliferation assays were performed. Each cell population was administered [³H]-thymidine, stimulated with antigenic peptides (10, 15, 7.5, and 3.8 µg/ml) and controls, and incubated for 24 h. Radioactivity incorporation compared to controls was taken as an indication of the expansion of the cell population in response to antigenic peptide stimulation. Figure 3 indicates, as expected, that PLN lymphocytes from C57BL/6J control mice are not stimulated by the antigenic peptides. Mice immunized with 0.1 µg of the TLR-7-ligand PSMA-PADRE conjugate (Figure 4) appear to have mounted an immune response,

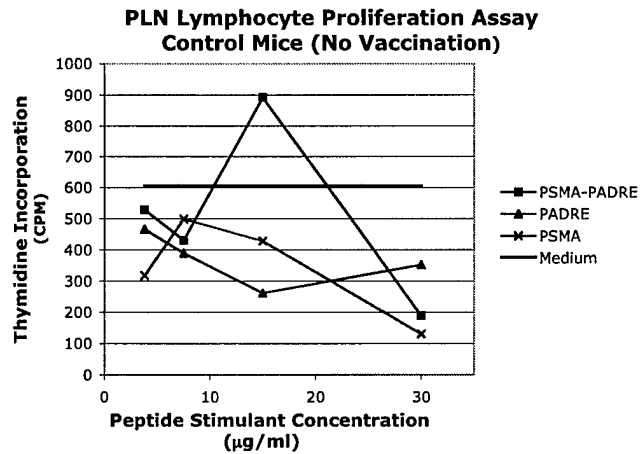


Figure 3. Non-transgenic control mice: No immune stimulation

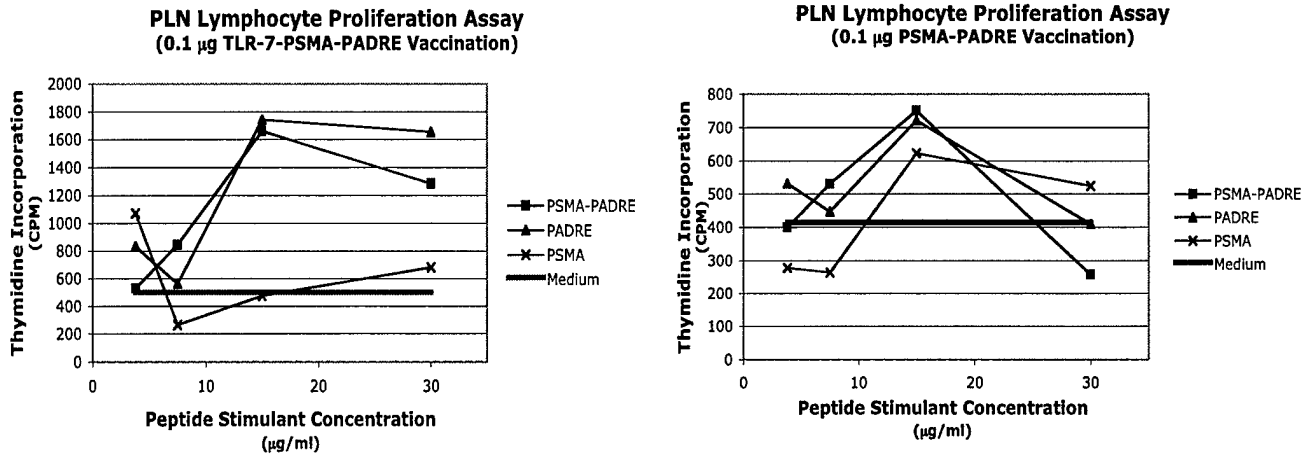


Figure 4. Transgenic mice, 0.1 µg vaccinations: Induction of an immune response

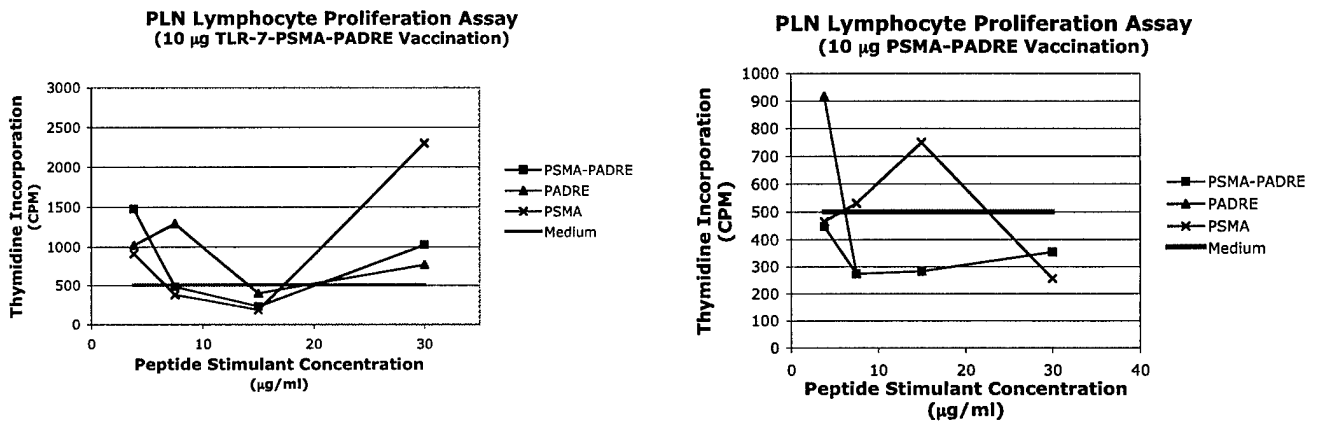


Figure 5. Transgenic mice, 10 µg vaccinations: Induction of tolerance

although apparently mainly to the PADRE epitope. Based upon thymidine incorporation levels, the TKL-7 conjugate induces an approximately 2.5X increase in response over that elicited from immunization with the PSMA-PADRE peptide alone. Interestingly, vaccination with 10 µg of the conjugate (Figure 5) appears to have resulted in tolerance to the antigens. Similar, although less clear-cut, results were obtained from the splenocyte assays (data not shown). All the data are being further refined.

Preliminary chromium release experiments evaluating cytotoxic T lymphocyte killing of LNCaP prostate cancer cells (which express the PSMA protein) in culture have not yet yielded any results. In preparation for these experiments, we have experienced difficulty with the uptake (or retention) of ⁵¹Cr by the LNCaP cells using standard labeling protocols. This technical problem should be solved shortly. Also, additional mouse immunization experiments with the TLR-7 conjugates in Figure 2 are underway.

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**)

In view of our difficulties with the PSA protein conjugates, we have replaced the PSMA protein-conjugate work with relevant PSMA peptide epitope experiments. These conjugates have been synthesized (Figure 2) and their evaluation has been incorporated into **Task 2** and is underway.

C. Key Research Accomplishments

This section provides a list of key accomplishments in the second year of this research.

- Functionalized 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 and TLR-2 ligand-antigenic peptide conjugates have been prepared as well as a TLR-7-ligand PSA protein conjugate.
- Mouse immunization experiments have begun. Initial data indicate an immunogenic response to the antigenic epitopes.

D. Reportable Outcomes

This program supports graduate research assistant, Mr. Jiang Sha, 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 being evaluated 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 a host immune response to prostate cancer antigens using the potent immune system activation that arises from the innate/adaptive response connection from 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

NAME HINSHAW, JERALD CLYDE		POSITION TITLE Research Associate Professor	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing. Include postdoctoral training.)</i>			
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: **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. A few are listed.

- P. Y. Lum, C. D. Armour, S. B. Stepaniants, G. Cavet, A. Leonardson, P. Garrett-Engele, M. K. Wolf, L. Butler, C. M. Rush, M. Bard, J. C. Hinshaw, P. Garnier, G. D. Prestwich, G. Schimmack, J. W. Phillips, C. J. Roberts, and D. D. Shoemaker, "Discovering Novel Modes of Action for Therapeutic Compounds using a Genome-wide Screen of Yeast Heterozygotes," *Cell*, 2004, **116**, 121-137.
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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