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14. ABSTRACT
The goals of this work are to develop engineered antibody fragments (cys-diabodies and cys-minibodies) for labeling with positron-emitting radionuclides and fluorescent dyes for multimodal imaging of prostate cancer. Previously, a novel cys-minibody recognizing PSCA (prostate stem cell antigen) was produced, conjugated, and radiolabeled with either I-124 or Zr-89. Targeting, imaging, and biodistribution studies in mouse models of prostate cancer confirmed excellent targeting and immunoPET imaging in PSCA-positive tumors in mice. This year, dual-labeling (with radionuclide and fluorescent dye) of the cys-minibody has been developed and evaluated in three different mouse models of prostate cancer. Furthermore, F-18 labeling has been established for a PSCA-specific cys-diabody and successfully imaged. A novel multifunctional linker has also been developed and dual F-18 PET/NIR fluorescence imaging has been achieved.

15. SUBJECT TERMS
Prostate cancer, imaging, antibody fragment, Positron emission tomography, fluorescence imaging, PSCA

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1. INTRODUCTION

Imaging remains a major unmet need in the management of prostate cancer. We are developing imaging probes based on engineered antibodies that recognize PSCA (prostate stem cell antigen), a cell surface protein highly expressed in prostate cancer. These engineered antibody fragments (cys-minibodies and cys-diabodies) can be labeled with radioisotopes for non-invasive PET imaging for use at multiple points in the prostate cancer treatment continuum, including staging at diagnosis, monitoring treatment, and re-staging at various points during management. Engineered fragments can also be labeled with fluorescent dyes for visual guidance in an intraoperative setting to ensure complete resection with negative margins. In this project, dually-labeled PSCA imaging agents are being developed that can be used for pre- and intra-operative detection of prostate cancer.

2. KEYWORDS

Prostate cancer, imaging, antibody fragment, positron emission tomography, fluorescence imaging, PSCA

3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1. Develop universal optimized cys-diabody and cys-minibody fragments against PSCA for PET imaging of prostate and pancreatic cancer.
Subtasks:

Major Task 1. Develop and evaluate cys-diabody and cys-minibody fragments
Major Task 2. Design, optimize and test multifunctional, F-18, and alternatively labeled fragments
Major Task 3. New technologies: alternative site-specific labeling methods, use of click chemistry

Specific Aim 2. Evaluate the ability of lead PSCA fragments to imaging prostate cancer in disease progression in xenograft and genetically engineered models of prostate cancer
Subtasks:

Major Task 4. Image bone and lymph node in xenograft models
Major Task 5. Image transgenic mouse models
Major Task 6. Development and evaluation of singly labeled and optimized optical probes for surgery
Major Task 7. Development of dual labeled probes for PET and optical imaging.

For Dr. Wu (Partnering PI) during Year 1 of the project, the subtasks were:
Major Task 1

Subtask 1. Produce and purify A2 cys-diabody and A2-cys-minibody in mg quantities (months 1-6) **Completed.**

Subtask 2. Optimize radiolabeling conditions with I-124 (tyrosine) and Zr-89 (DFO conjugation to lysine and cysteine residues). Confirm retention of binding by QCM and cell binding. (months 3-9) **Completed.**

Subtask 3. Conduct microPET imaging and biodistribution in subcutaneous models using I-124 and/or Zr-89; provide PET tracers to Aim 2. (months 6-18) **Completed and ongoing.**

For Partnering PI Dr. Wu, during Year 2 of the project, the subtasks were:

Major Task 2: Design, optimize, and evaluate cys-diabody and cys-minibody fragments.

Subtask 1: Optimize dual-labeling of cys-minibody and cys-diabody and confirm targeting and imaging in subcutaneous models. (months 12-18) **Completed.**

Subtask 2: Develop and establish cysteine specific F-18 labeling of A2 cys-diabody. (months 12-18) **Completed**.

Subtask 3: Conduct microPET imaging and biodistriubtion in subcutaneous models using F-18 cys-diabody. (months 14-24) **In progress**.

Subtask 4: Produce mannosylated proteins and develop cold chemistry for conjugation. (months 18-30) **Discontinued** previously due to lack of feasibility. Focus shifted to multifunctional linkers.

Subtask 5. Design multifunctional linkers, establish and optimize conjugation conditions. (months 12-36) **In progress.**

Subtask 6. Continue to provide PET, optical, and dual-labeled probes to Aims 2 and 3. (months 1-36) **Ongoing**.

Major Task 3: New technologies

Subtask 1: Focus on alternative approaches including radiolabeling mannosylated cys-diabody and cys-minibody. **Discontinued** previously due to poor labeling efficiency. Efforts shifted to multifunctional linkers.

Subtask 2: Continue work on multifunctional linkers to produce labeled cys-diabody with "click" handle. (months 12-36) **In progress**.

Subtask 3. Work on "click" F-18 radiolabeling of multifunctionally modified cys-diabody. (months 24-36) **In progress**, ahead of schedule.

Major Task 4: Image bone and lymph node in xenograft models

Subtask 1: Image bone implant/metastatic models with best approach labeling cys-diabody and cys-minibody. (months 6-24) **In progress**.

Major Task 7: Development of dual labeled probes for PET and optical imaging

Subtask 1: Dual labeling for optical/PET imaging. (months 24-36) **In Progress**, ahead of schedule.

Subtask 2: In vivo dual labeling in xenograft and possibly transgenic model systems. (months 24-36) **In progress**, ahead of schedule.

What was accomplished under these goals?

Major Task 2: Design, optimize and evaluated cys-diabody and cys-minibody fragments

Subtask 1. Optimize dual-labeling of cys-minibody and cys-diabody and confirm targeting and imaging in subcutaneous models.

ImmunoPET/Fluorescence Imaging of PSCA-Positive Prostate Cancer Using A11 Cys-Minibody

Wen-Ting K. Tsai[1], Kirstin A. Zettlitz[1], Richard Tavaré[1], Robert E. Reiter[2], and Anna M. Wu[1]
[1]Dept of Molecular and Medical Pharmacology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
[2]Dept of Urology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Current challenges in prostate cancer imaging include the need to detect and determine the extent of disease pre-operatively and intra-operatively. Prostate stem cell antigen (PSCA) is a cell-surface marker overexpressed in primary and metastatic cancers[1]. *In vivo* administration of radiolabeled antibody fragments specific for PSCA can be used for immunoPET imaging with high target-to-background at short imaging times post-injection[2]. An anti-PSCA antibody fragment can also be used to fluorescently identify PSCA-positive tumors indiscernible by white light[3].

In order to facilitate production of a dually labeled PSCA fragment for PET/optical imaging, the A11 minibody was modified with a C-terminal cysteine (A11 cMb) for site-specific conjugation. Maleimide-Cy5.5 conjugated A11 cMb retained low nanomolar affinity (13 nM) for PSCA-positive cells. Radiolabeling of the A11 cMb was accomplished using Iodogen (for ^{124}I) or conjugation to SCN-desferrioxamine (DFO) (for ^{89}Zr) with retention of immunoreactivity (>70%). Dually labeled ^{124}I-A11 cMb-Cy5.5 successfully targeted s.c. 22Rv1xPSCA human prostate cancer tumors in nude mice by PET, with positive tumor (11.7 ± 1.3%ID/g) and 22Rv1 control tumor (1.2 ± 0.6%ID/g) uptake quantified by *ex vivo* biodistribution. The ^{124}I-A11 cMb-Cy5.5 biodistribution was similar to the singly labeled ^{124}I-A11 cMb, resulting in positive-to-negative tumor ratios of 13:1 and 8:1, respectively. Fluorescent imaging post-mortem with the skin removed showed a strong signal in 22Rv1xPSCA tumors compared to control tumors. In a second human prostate cancer model, ^{124}I-A11 cMb-Cy5.5 targeted s.c. PC3xPSCA tumors by PET with specific uptake in positive tumors (1.8 ± 0.5%ID/g) in comparison to control tumors (0.5 ± 0.1%ID/g) as quantified by biodistribution, resulting in a positive-to-negative tumor ratio of 4:1. The moderate overall tumor uptake could be due to a lower PSCA cell-surface density of 500,000/cell for PC3xPSCA in comparison to 2.2x10^6/cell for 22Rv1xPSCA. Fluorescence signal in the *ex vivo* positive tumors was higher in comparison to other resected tissues. In an orthotopic model, ^{89}Zr-A11 cMb-Cy5.5 targeted intraprostatically implanted 22Rv1xPSCA tumors by PET with 3.1 ± 0.5%ID/g uptake, and fluorescence clearly distinguished the prostate tumor from surrounding seminal vesicles. In conclusion, the A11 cMb can be site-specifically conjugated with a fluorescent dye and radiolabeled to specifically target PSCA tumor burden by immunoPET/fluorescence. This humanized probe has the potential to pre-operatively detect a patient's primary and metastatic prostate cancer and guide surgical resection in real time.

(a) (b) (c)

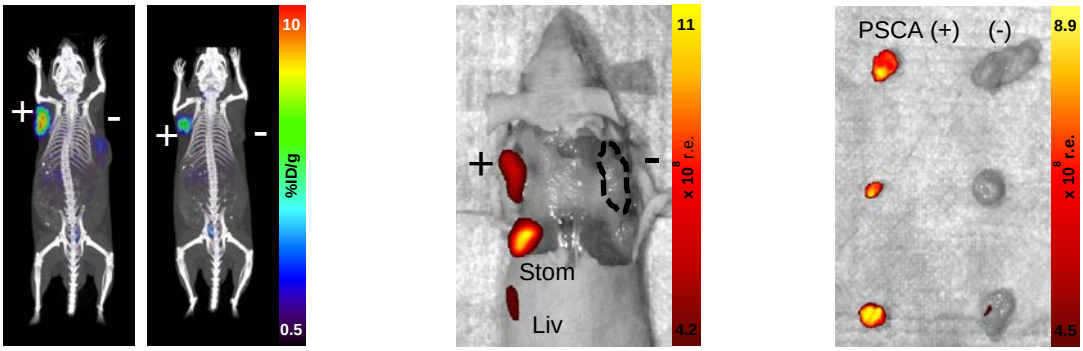
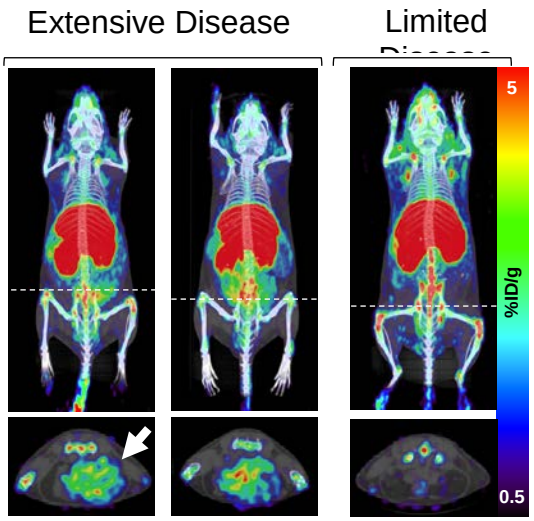


Figure 1. ^{124}I-A11 cMb PET/CT (whole body MIP) scans of 2 representative nude mice bearing 22Rv1xPSCA (left shoulder) and 22Rv1 (right shoulder) tumors at 22 hours post-injection results in high PSCA-positive tumor uptake (a). Subsequent Cy5.5 fluorescent imaging *in situ* (b) and *ex vivo* (c) specifically image PSCA-positive tumors with some uptake in the liver and autofluorescence signal from stomach.



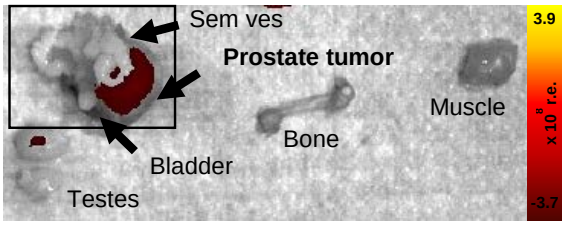


Figure 2. ^{89}Zr-A11 cMb-Cy5.5 was injected in nude mice bearing extensive or limited 22Rv1xPSCA orthotopic tumors, and PET/CT images were obtained at 22 hours post-injection (Top row – whole body coronal view, 30 mm MIP; bottom row – transverse view, 0.2 mm) (a). Prostate tumor and other resected tissues imaged by Cy5.5 fluorescence to show specific signal in the prostate and little to no signal in

Subtask 2: Develop and establish cysteine specific F-18 labeling of A2 cys-diabody

The A2 cys-diabody is the focus of this work, since the smaller diabody fragments target and clear quickly enough to be coupled with F-18 for rapid imaging. A linker for site-specific conjugation via maleimide chemistry and enabling "click"-chemistry of ^{18}F-TCO to the tetrazine was successfully synthesized. It was conjugated to the C-terminal Cys-tag of A2 cys-diabody (A2cDb) and used for immunoPET imaging of nude mice bearing PSCA positive (22Rv1-PSCA) and PSCA negative (22Rv1) prostate cancer xenografts. Specific tumor uptake is visible as early as 1 hour postinjection.

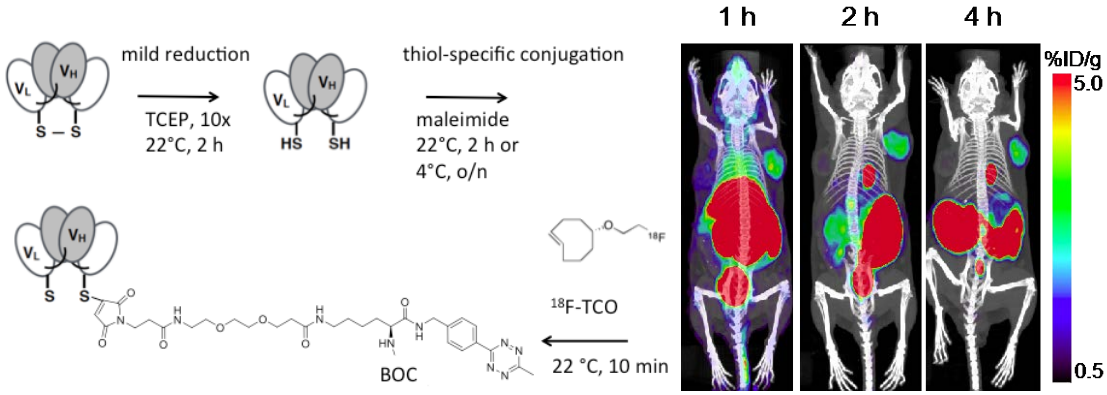


Figure 3. A2cDb-malPEG-TZ-TCO-F18, 22Rv1-PSCA tumor on the right shoulder and 22Rv1 on the left shoulder.

Subtask 5: Design multifunctional linkers

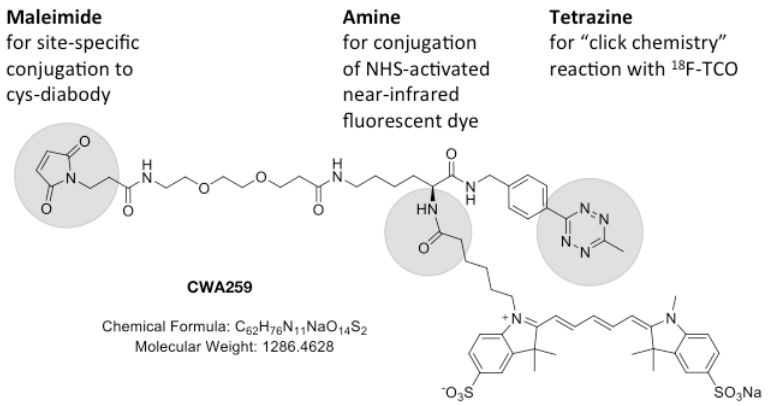


Figure 4. Multifunctional linker design.

Above is an example of the multifunctional linkers that have been designed for producing dually-labeled PET/optical probes. The linker includes a maleimide group for site-specific conjugation to cys-diabodies, an amine group for conjugation to any of multiple fluorescent dyes using NHS chemistry, and a tetrazine moiety for "click chemistry."

Major Task 3: New technologies

Subtask 2: Continue work on multifunctional linkers to produce fluorescently labeled cys-diabody with "click" handle

Subtask 3: "click" F-18 radiolabeling of multifunctionally modified cys-diabody

A2cDb was modified with a dual-modality linker, allowing for click-labeling of TCO-F-18 and containing the near-infrared fluorescent dye sulfo Cy5 for optical imaging.

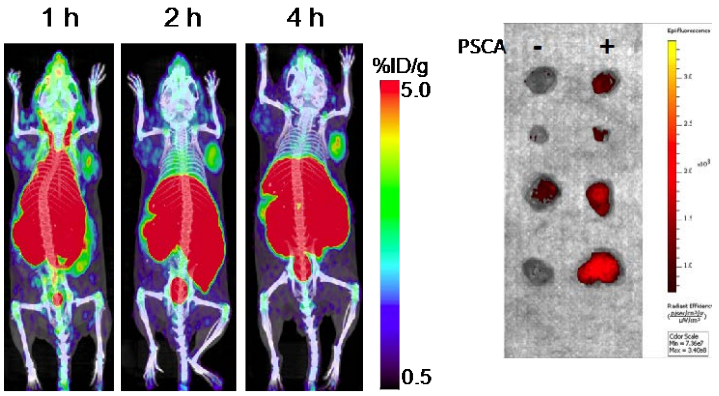


Figure 5. A2cDb-malPEG-sCy5-TZ-TCO-F18 imaging of 22Rv1-PSCA xenografts (right shoulder) and 22Rv1 (left shoulder)

The proof-of-concept images above demonstrate the ability to detect tumors by immunoPET within 1-4 h of administration of the dual-labeled probe, and subsequent ex vivo confirmation of localization of fluorescence in the PSCA-positive tumors.

Major Task 7

Subtask 2. In vivo dual labeling experiments in xenograft and possibly transgenic model systems.

See results above for progress developing and evaluating an F-18/sulfoCy5 dual-labeled cys-diabody. Detailed characterization is in progress.

In parallel, the cys-minibody has been dual-labeled with either Zr-89 or I-124, and Cy5 for studies in three models of prostate cancer. See results in Major Task 2, Subtask 1, described above.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

During the next year we will continue to evaluate our lead dually-labeled PET/optical probes in more biologically relevant models of prostate cancer including bone and lymph node metastasis models as well as genetically engineered mouse models, in close collaboration with the overall PI, Dr. Robert Reiter. We will also apply these in intraoperative models of prostate cancer, again in with Dr. Reiter. Finally, we will continue to work on the dual F-18/optical probes based on the smaller A2 cys-diabody including development and use of novel multi-functional linkers.

4. IMPACT

What was the impact on the development of the principle discipline of the project?

Nothing to report.

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. After only one year of funded research it is too early for our findings to have significant impact.

5. CHANGES/PROBLEMS

Nothing to report. There are no significant changes to the objectives, scope, and approaches of the project.

6. PRODUCTS

Publications, conference papers, and presentations

Abstracts

Tsai, W., Zettlitz, K., Tavaré, R., Salazar, F., Reiter, R., and Wu, A. (2017) Dual-modality immunoPET/fluorescence imaging of prostate cancer utilizing ^{89}Zr- or ^{124}I-anti-PSCA cys-minibody. American Association for Cancer Research Annual Meeting; 2017 April 1-5. Washington, DC.

Tsai, W.-T.,K., Zettlitz, K.A., Tavaré, R., Reiter, R.E., and Wu, A.M. (2017) ImmunoPET/fluorescence imaging of PSCA-positive prostate cancer using A11 cys-minibody. World Molecular Imaging Congress, Philadelphia, PA.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Anna Wu*
Project Role:	Partnering Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Wu oversaw all aspects of work performed and accomplished to-date.*
Funding Support:	

Name:	*Jennifer Murphy*
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Murphy had performed work on the design and evaluation of the chemical and radiochemical strategies for combined PET/optical labeling.*
Funding Support:	

Name:	*Kirstin Zettlitz*
Project Role:	Assistant Researcher
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*4*
Contribution to Project:	*Dr. Zettlitz had performed work on site-specific conjugation, radiolabeling, and imaging of engineered antibody fragments.*
Funding Support:	

Name:	*Maruthi Narayanam*
Project Role:	Postdoctoral Researcher
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Narayanam had performed work on developing methods for F-18 labeling of engineered antibody fragments.*
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

New funding: R21 CA212718 High-Throughput Radiochemistry Platform for Accelerated Discovery and Development of Novel PET Imaging Agents for Cancer (van Dam, PI); time commitment of 0.36 calendar months; project period of 4/01/2017 – 3/31/2019

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Collaborative awards

This report covers the activities of the Partner PI, Dr. Anna Wu.

9. APPENDICES

None