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TITLE: Development of Novel PD1/PD-L1 Antagonists Using Circular Cys-Knotted Micro Proteins

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

During these two years we have also accomplished the design and expression of a FRET-based reporter to screen antagonists for the PD-1/PD-L1 complex. We have constructed and screened genetically-encoded libraries using the loops1 and 6 of cyclotide MCoTI-I. This library was screened and a bioactive cyclotide, MCo-101B, was selected. This cyclotide was able to inhibit the PD-1/PD-L1 with an IC50 value of 0.66 μM. This exciting finding represents the first cyclotide selected by molecular evolution that can inhibit the PD-1/PD-L1 complex with sub-μM activity. This cyclotide has been used to perform preliminary toxicology studies, not showing toxicity in mice with dosing up 10 mg/kg. The cyclotide is being tested for efficacy in vivo in a lung cancer syngeneic model in mice.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. **INTRODUCTION:**

Carcinoma of the lung is one of the most common types of cancer worldwide. Lung cancer causes more deaths than the next three most common cancers combined (colon, breast and pancreatic). It is estimated that just in the United States around 160,000 people are expected to die from lung cancer in 2015, accounting for approximately 27% of all cancer deaths. This highlights the need for more effective therapies to treat this type of lethal disease. Increasing evidence shows that tumors can evade adaptive immunity and disrupt T-cell checkpoint pathways. The interaction between the PD-1 receptor and its ligands PD-L1 and PD-L2 is a key pathway hijacked by tumor cells to evade immune control. Hence, reversing the inhibition of the adaptive immunity can lead to the activation of a patient's immunity. For example, inhibition of the checkpoint pathways should block tumor immune inhibition. To date, several antagonistic mAbs have developed against the cytotoxic CTLA-4, the PD-1 receptor and PD-L1, to block immune checkpoints, and facilitate antitumor activity. These checkpoint-blocking antibodies have demonstrated clinical activity in a variety of tumor types, including melanoma, renal cell carcinoma, and lung cancer. Lung tumor cells have been found to express PD-L1 allowing the tumors to directly suppress anti-tumor cytolytic T cell activity and T cell down-regulation and inhibition. Blocking the interaction of the PD-1 receptor with one of its ligands, PD-L1, using mAbs has shown to increase the T cell response against the tumor. Two clinical trials involving the use of therapeutic mAbs able to block the PD-1/PD-L1 pathway have shown very promising results in lung cancer.

Objectives. Therapeutic mAbs are the fastest growing class of new therapeutic molecules. They hold great promises for the treatment of a variety of diseases, including cancer and chronic inflammatory diseases. However, the current manufacturing and purification processes cause limitations in the production capacity of therapeutic antibodies, leading to an increase in cost. We propose to use a micro-protein-based molecular scaffold (also cyclotide) for generating molecular libraries that will be screened and selected for potential antagonists for the PD-1/PD-L1 interaction. These compounds will be then screened and selected for their ability to antagonize the interaction between PD-1 and PD-L1 inside the bacterial cell using a genetically-encoded FRET-based reporter. We will use high throughput flow cytometry to identify bacteria encoding cyclotides able to specifically disrupt the soluble PD-1/PD-L1 complex. Selected cyclotides will be structurally characterized by NMR and assayed *in vitro* first to evaluate their ability to bind cells expressing PD-L1 and to antagonize the PD-1/PD-L1 pathway. Cyclotides with potent *in vitro* PD1/PD-L1 inhibitory properties will be further tested *in vivo* using immunocompetent syngeneic mouse models of lung cancer.

Specific Aims

Specific Aim 1. To screen and select cyclotide-based peptides able to disrupt the PD-1/PD-L1 interaction. The objectives of this aim are 1) the production of large genetically-encoded libraries of cyclotides and 2) the production of cellular FRET-based screening

reporter to select cyclotides able to inhibit PD-1/PD-L1. Cells able to express active cyclotides will be selected using high throughput flow cytometry methods such as fluorescence activated cell sorting (FACS).

Specific Aim 2. To test and evaluate the inhibitory and biological activity of selected cyclotides *in vitro*. The objectives of this aim are 1) test selected cyclotides *in vitro* using a combination of fluorescence assays and nuclear magnetic resonance (NMR) and 2) evaluate their ability to block the PD1/PD-L1 pathway activity.

Specific Aim 3. To evaluate *in vivo* efficacy of the most potent cyclotide as a single agent. The objectives of this aim are to 1) evaluate *in vivo* efficacy and 2) toxicity of the most promising cyclotide. This will be accomplished using an immunocompetent syngeneic mouse model of lung cancer, and bioactive cyclotides.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Cyclotides, microproteins, immunotherapy, immune checkpoint, PD-1, PD-L1

3. **ACCOMPLISHMENTS:**
    - **What were the major goals of the project?**
        - *Major Task 1. Develop a cell-based genetically encoded FRET-based reporter to screen antagonists for the PD-1/PD-L1 complex.*
        - *Major Task 2. Creation of cyclotide-based libraries using the MCoTI-cyclotide molecular scaffold in E. coli.*
        - *Major Task 3. Screen libraries of circular peptides inside E. coli cells to select potential cyclotides able to antagonize the PD-1/PD-L1 complex.*
        - *Major Task 4. In vitro characterization of selected cyclotides able to antagonize PD-1/PD-L1.*
        - *Major Task 5. Evaluate in biological activity of bioactive cyclotides in syngenic mouse models of lung cancer.*
    - **What was accomplished under these goals?**
        - ***Major Task 1. Develop a cell-based genetically encoded FRET-based reporter to screen antagonists for the PD-1/PD-L1 complex****. We have developed a FRET-reporter for the PD-1/PD-L1 interaction. We used the CyPet and YPet fluorescent proteins as a FRET-couple to monitor the interaction between PD-1 and PD-L1. To facilitate the interaction between targeted domains and prevent any steric hindrance that will interfere with the molecular recognition process, we used an appropriate flexible polypeptide linkers (i.e. $[GGS]_5$) at the junctions between the interacting extracellular protein domains and the corresponding fluorescent proteins. Briefly, the extracellular domain of murine PD-L1 (residues 18-239, with a Met added to the N-terminus) and murine PD-1 (residues 25-157, with unpaired Cys83 mutated to Ser and a Met-Ala added to the N-terminus) were fused to C-terminal of CyPet and YPet, respectively. Please note the N-fusions resulted in poor yield expression (**Fig. 1**) We have also developed a poly-cistronic expression plasmid for the co-expression of both fluorescent proteins, PD-1 and PD-L1, in E. coli cells to perform in-cell screening of inhibitors against the PD-1/PD-L1 complex.*
        - ***Major Task 2. Creation of cyclotide-based libraries using the MCoTI-cyclotide molecular scaffold in E. coli.*** *We have produced a generically encoded library using the loops 1 and 6 of cyclotide MCoTI-I containing around 10 billion different sequences. This library was created* at the DNA level using double stranded DNA inserts with degenerate sequences for loops 1 and 6 of cyclotide MCoTI-I. Briefly, a long degenerate synthetic oligonucleotide encoding the whole cyclotide, ≈100 nucleotide-long template is PCR amplified using 5'- and 3'-primers corresponding to the non-degenerate flanking regions. The resulting double-stranded degenerate DNA was double digested and then ligated to a linearized intein-encoding expression vector to produce a library of pASK-based plasmids. These libraries were then transformed into electrocompetent *E. coli* cells previously transformed with the FRET-based reporter to

finally obtain a library of cells typically containing up to ≈10^9 different clones (i.e. cyclotide sequences).

- ***Major Task 3. Screen libraries of circular peptides inside E. coli cells to select potential cyclotides able to antagonize the PD-1/PD-L1 complex****. Using the Scheme shown in* ***Fig. 2*** *we have been able to perform the first in-cell screening of PD-1/PD-L1 cyclotide based antagonists. Preliminary screening assays of this library have already yielded a sub-μM cyclotide-based PD-1/PD-L1 antagonist, cyclotide MCo-101B (****Fig. 3****).*
-
- ***Major Task 4. In vitro characterization of selected cyclotides able to antagonize PD-1/PD-L1.*** *Using our FRET-based reported we have evaluated the IC_{50} of cyclotide cyclotide MCo-101A (****Fig. 2****).*
- ***Major Task 5. Evaluate in biological activity of bioactive cyclotides in syngenic mouse models of lung cancer.*** *This part is being completed by our collaborator Dr. Neamati at University of Michigan. We have started the toxicology studies showing the cyclotide MCo-101B is not toxic in mice up to doses of 10 mg/kg (****Fig. 4****). The efficacy of cyclotide MCo-101B is still being evaluated in a syngeneic mouse model of lung cancer. Problems associated with the large-scale synthesis of MCo-101B delayed the original plan to have finished the studies before the end of the award. This work is still being completed.*

- **What opportunities for training and professional development has the project provided?**
  - *Nothing to report.*
- **How were the results disseminated to communities of interest?**
  - *Some of the results/technologies developed in this proposal have been disseminated in conferences and peer-reviewed reviews.*
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - *Nothing to report.*

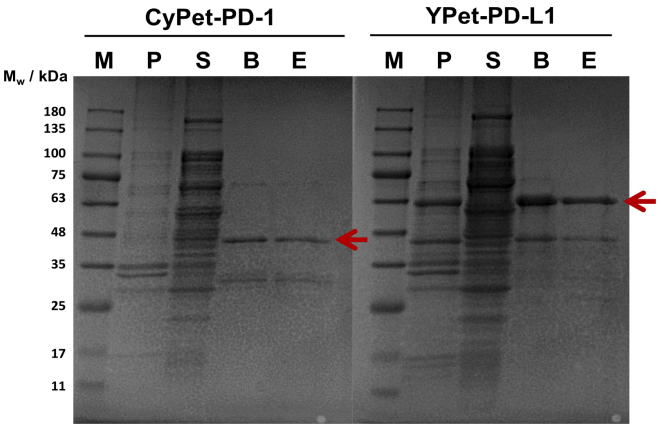

Fig. 1 Expression, purification and characterization of CyPet-PD-1 and YPet-PD-L1 in E. coli cells. These proteins were used to test the in vitro activity of cyclotide MCo-101A as shown in **Fig. 3**. M: markers, P: insoluble cell lysate fraction, S: soluble cell lysate fraction, B: pulldown with Ni^{2+}-NTA-sepharose beads, and E: purified protein.

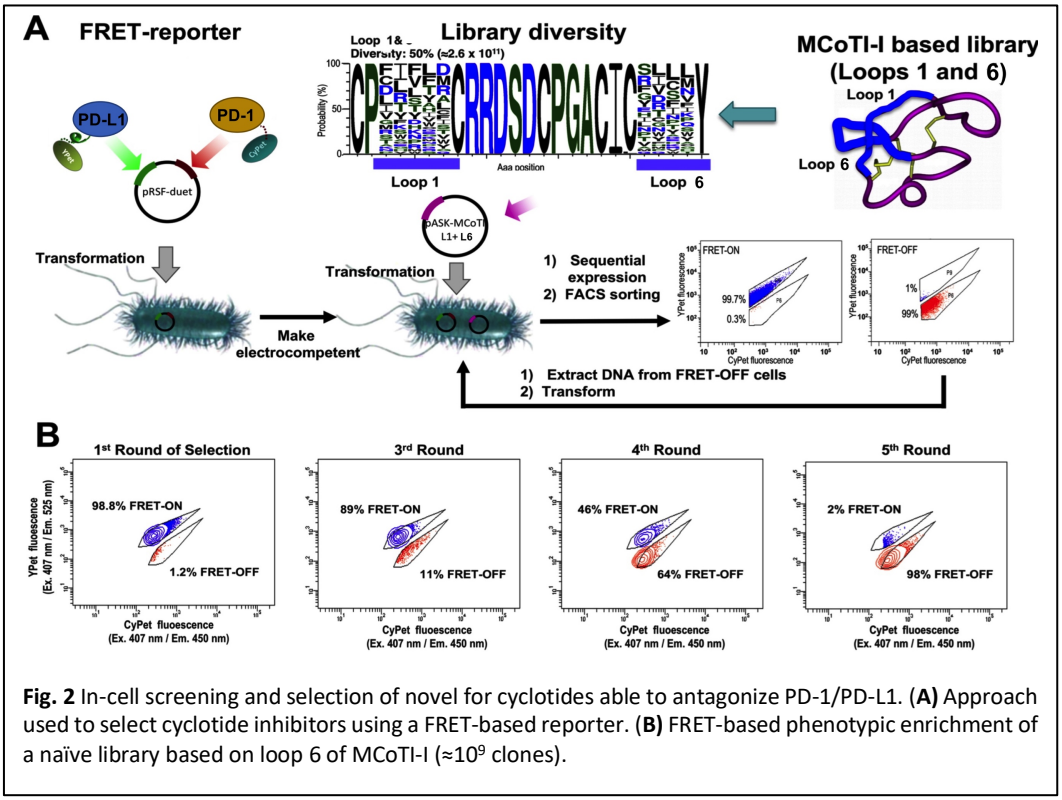

Fig. 2 In-cell screening and selection of novel for cyclotides able to antagonize PD-1/PD-L1. (**A)** Approach used to select cyclotide inhibitors using a FRET-based reporter. (**B)** FRET-based phenotypic enrichment of a naïve library based on loop 6 of MCoTI-I (≈10^9 clones).

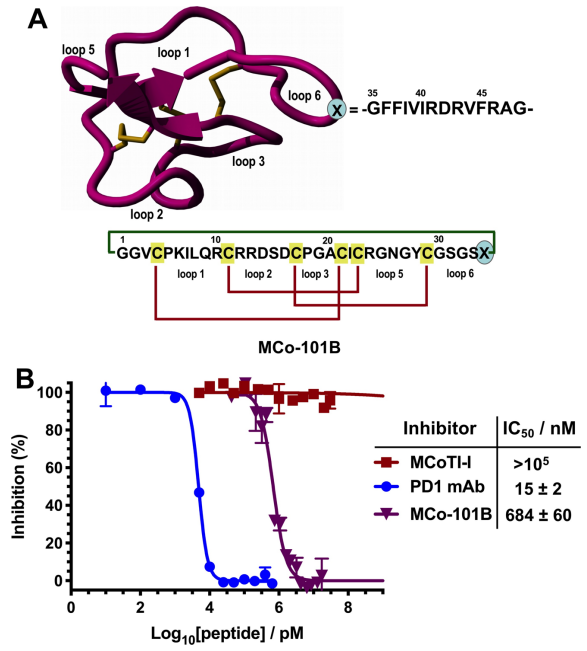

Fig. 3 Novel cyclotide MCo-101B inhibits the interaction between PD-1 and PD-L1. **A.** Sequence of cyclotide MCo-101B. B**.** Cyclotide MCo-101B specifically antagonizes the binding of the extracellular domains of PD-1 and PD-L1. The human anti-PD-1 mAb (Cytocares GMP-A085) and cyclotide MCoTI-I were used as positive and negative controls. See text for description of the assay and fluorescent proteins.

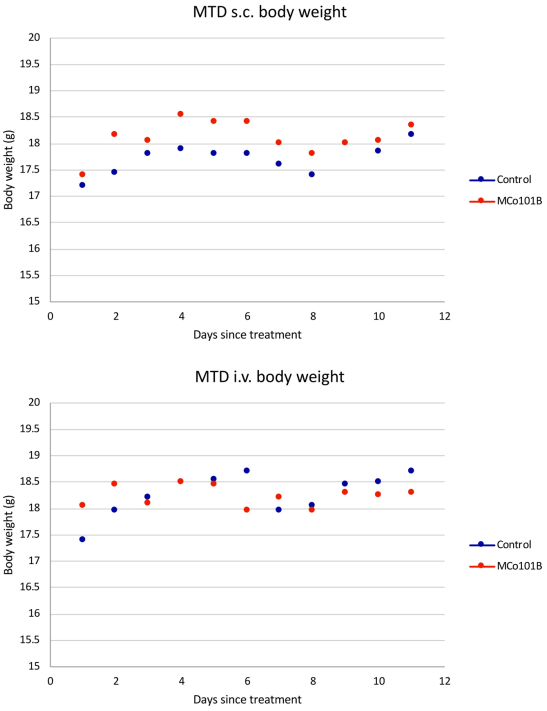

Fig. 4 Maximum tolerated dose (MTD) studies for bioactive cyclotide MCo-101B in immunocompetent C57BL/6 mice using daily intravenous (IV) and subcutaneous (SC) dosing at 10 mg/kg for 11 days.

4. **IMPACT:**
    - **What was the impact on the development of the principal discipline(s) of the project?**
        - *We have developed for the first the production of genetically-encoded libraries of cyclotides. These libraries, containing billions of difference micriproteins, would provide a source of cyclotides to antagonize PD-1/PD-L1 complex but also other pharmacologically relevant cancer validated molecular targets.*
        - *The development of a FRET-based screening system to select PD-1/PD-L1 antagonist could be used also for in vitro high throughput screening of protein, peptides and small molecules.*
    - **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**
        - *Nothing to report.*
    - **Actual or anticipated problems or delays and actions or plans to resolve them**
        - *We found some delays on the designing of the PD-1 and PD-L1 fluorescent based reporter due to initial problems with the solubility of these proteins when expressed in E. coli. This was solved by fusing the fluorescent protein to the N-terminal of the extracellular domains of PD-1 and PD-L1.*
        - *Our final screens have yielded a sub-µM inhibitor (IC_{50} ≈ 0.6 µM) (**Fig. 3**). This cyclotide has not shown toxicity at daily doses of up to 10 mg/kg for 11 days (**Fig. 4**). We are in the process of testing the efficacy of this cyclotide in a TLLC1 syngeneic mouse model of lung cancer. The delay for the efficacy studies have be Delays in this have been caused by some difficulties (already solved) when scaling up the synthesis of the cyclotide MCo-101B.*
    - **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**
        - *No human subjects were involved in this work.*
    - **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**
        - **Journal publications.**
        -
            - *1) M. J. Cambell, J. Su and **J. A. Camarero** (2019) Recombinant production of cyclotides using expressed protein ligation (EPL), Methods Mol. Biol., in press.*
            - *DoD-CDMRP acknowledged: Yes.*
            - *URL: not available yet.*
            - *2) **J. A. Camarero** and M. J. Campbell (2019) The potential of the cyclotide scaffold for drug development, Biomedicines, **7**(31), 1-20.*
            - *DoD-CDMRP acknowledged: Yes.*
            - *URL: www.ncbi.nlm.nih.gov/pubmed/31010257*
            - *3) C. Sarmiento and **J. A. Camarero** (2019) Biotechnological applications of protein splicing, Curr. Protein Pept. Sci., **20**(5), 48-424.*
            - *DoD-CDMRP acknowledged: Yes.*
            - *URL: www.ncbi.nlm.nih.gov/pubmed/30734675*
            - *4) **J. A. Camarero** (2017) Cyclotides, a versatile ultrastable micro-protein scaffold for biotechnological applications, Bioorg. Med. Chem. Lett., **27**(23), 5089-5099.*
            - *DoD-CDMRP acknowledged: Yes.*
            - *URL: www.ncbi.nlm.nih.gov/pubmed/29110985*
            - *5) A. Gould and **J. A. Camarero** (2017) Cyclotides: Overview and biotechnological applications, ChemBiochem, **8**(14), 1350-1363. DoD-CDMRP acknowledged: Yes.*
            - *URL: www.ncbi.nlm.nih.gov/pubmed/28544675*
            - *6) K. Jagadish and **J. A. Camarero** (2017) Recombinant expression of cyclotides using split inteins, Methods Mol. Biol., **1495**, 41-55. DoD-CDMRP acknowledged: No.*
            - *URL: www.ncbi.nlm.nih.gov/pubmed/27714609*
        - **Oral Presentations**
            - *1) Invited talk to the Drug Discovery Chemistry 2018 – Macrocyclics & Constrained Peptides: Rapid Screening of Cyclotide-Based Libraries against Intracellular Protein-Protein Interactions, April 5, 2018 in San Diego, CA*
            - *DoD-CDMRP acknowledged: Yes.*
            - *2)Invited talk to 13th Enzymes in Drug Discovery Summit: Using the cyclotide scaffold to target protein-protein interactions, February 22, 2018 San Diego, California. DoD-CDMRP acknowledged: Yes.*
            - *3) Invited talk PepTalk 2018 meeting – Recombinant Protein Expression and Production: Recombinant expression of circular Cys-knotted microproteins. Application for in-cell high throughput*

screening of specific protein-protein antagonists, January 10, 2018, San Diego, California. DoD-CDMRP acknowledged: Yes.

- *4) Invited oral presentation to the seminar series at the Department of Chemistry, Boston College: Using the cyclotide molecular scaffold to target protein-protein interactions, November 29, 2017, Boston, Massachusetts. DoD-CDMRP acknowledged: Yes.*
- *5) Invited talk to Novartis: Cyclotides, a new molecular scaffold to target protein-protein interactions, May 3, 2017, Cambridge, Massachusetts. DoD-CDMRP acknowledged: Yes.*
- *6) Invited talk to the 13th Annual PEGS at Boston 2017 – Protein Engineering stream: Rapid Screening of Cyclotide-Based Libraries against Intracellular Protein-Protein Interactions, May 1, 2017, Boston, Massachusetts. DoD-CDMRP acknowledged: Yes.*

- **Website(s) or other Internet site(s)**
  - *Nothing to report*
- **Technologies or techniques**
  - *1) Developed new high throughput FRET-based assay to screen libraries of compounds in cell or in vitro. 2) Generated genetically-encoded libraries of cyclotide MCoTI-I using loops 1 and/or 6.*
- **Inventions, patent applications, and/or licenses**
  - *Nothing to report yet.*
- **Other Products**
  - *Nothing to report.*

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**

Name:	*Julio A. Camarero*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-9150-0665*
Nearest person month worked:	*No change*
Contribution to Project:	*No change.*
Funding Support:	
Name:	*Nouri Neamati*
Project Role:	*coinvestigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-3291-7131*
Nearest person month worked:	*No change*
Contribution to Project:	*No change.*
Funding Support:	
Name:	*Jagadish Krishnappa*
Project Role:	*Postdoc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12 months*
Contribution to Project:	*Left research group on June 1st, he won't be contributing to the 2nd year, his role would be replaced by Dr. Corina Sarmiento.*
Funding Support:	
Name:	*Teshome Aboye*
Project Role:	*Postdoc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*No change*

Contribution to Project:	*No change.*
Funding Support:	
Name:	*Corina Sarmiento*
Project Role:	*Postdoc (to replace Dr. Jagadish Krishnappa)*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1 month*
Contribution to Project:	*1 month in first year . In the 2nd year she will contribute full time (100%)*
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?**
  - *Nothing to Report.*
- **What other organizations were involved as partners?**
  - *Nothing to Report.*

8. **SPECIAL REPORTING REQUIREMENTS**
    - **COLLABORATIVE AWARDS:** *No required..*
    - **QUAD CHARTS:** *No required.*

9. **APPENDICES:**