AWARD NUMBER: W81XWH-19-1-0509

TITLE: Targeting PLK-1 for Treating MYC-Driven Lymphomas

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Roswell Park Comprehensive Cancer Center, Buffalo, New York; and
University of Nebraska Medical Center,

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: We have previously shown that overexpression of Polo-like kinase (PLK)-1 is associated with poor clinical outcomes in patients with aggressive B cell lymphomas. We found that PLK-1 selective inhibitor, Volasertib, has a potent anti-tumor effect in aggressive B cell lymphomas *in vitro* and *in vivo*. Unfortunately, Volasertib was withdrawn in Phase III clinical trials due to its adverse effects probably related to myelosuppression. Herein, we developed a Volasertib antibody-drug conjugate (V-ADC) using CD19 antibody *Inebilizumab* in order to increase the targeting specificity for B-cell lymphoma cells and to minimize the side effects of Volasertib. However, our initial investigation found that V-ADC exhibited little cytotoxic effects, comparing to Volasertib treatment in Z138 cells, a mantle cell lymphoma cell line. CD19 has been known as a surface biomarker for normal and neoplastic B cell, however, we observed the level of CD19 expression in Z138 cells was relatively low. Furthermore, CD19 gene in Z138 carries a *P. L174V* mutation in exon 3 coding for extracellular domain. We thus hypothesized that the ability of antigen recognition by *Inebilizumab* could be compromised by low level CD19 and/or CD19 gene mutation. We thus established stable Z138 cell lines with overexpression of wild type CD19 (CD19-WT) and *L174V* mutant CD19 (CD19-Mut) to further study the cytotoxic effect of V-ADC. We treated these cells with 10 nM V-ADC for 48 h and the apoptotic subpopulations was determined by flow cytometry. We found that cells with CD19-overexpression, both wild type and mutant forms, exhibited markedly increased cell apoptosis comparing to parental cells (53-55% in overexpressing cells versus 5% in parental cells). In addition, V-ADC treatment significantly induced a reduction of mitochondria membrane potential (TMRE), activations of caspase 3 and 9, and cleavage of PARP in both overexpressing CD19-WT and -Mut Z138 cell lines. We did not identify any significant difference in V-ADC-induced cytotoxic effects between the CD19-WT and -Mut cells. In conclusion, the therapeutic efficacy of V-ADC depends heavily on the levels of CD19 expression and the gene mutation in the exon 3 of CD19 (*P. L174V*) might not have a significant impact in CD19-binding in our system. We are actively investigating the *in vivo* therapeutic effects of V-ADC in patient-derived xenograft (PDX) animal models of MCL and other aggressive B-cell lymphomas.

15. SUBJECT TERMS NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly describes the subject, purpose and scope of the research.*

PLK1 is a potential therapeutic target since PLK1 overexpressed in a variety of cancers, which linked to tumor development and poor clinical outcomes (Iliaki, Beyaert, & Afonina, 2021; Petronczki, Lénárt, & Peters, 2008). PLK1 overexpression plays an role in chemo-resistance, which was alleviated by PLK1 inhibition (Liu, Sun, & Wang, 2017). PLK-1 selective inhibitor, Volasertib, has a potent anti-tumor effect in B cell non- Hodgkin's lymphomas (B-NHL) (E. Chen & Pei, 2020; Hassan, Alinari, & Byrd, 2018; Murga-Zamalloa et al., 2017). Our lab has previously shown that Volasertib significantly suppressed tumor growth in a xenograft mouse model of double-hit lymphoma through promoting MYC degradation (Ren et al., 2018). Unfortunately, Volasertib was withdrawn from Phase III clinical trials due to its adverse effects, possible related to myelosuppression (Dill et al., 2020; Gjertsen & Schöffski, 2015; Schöffski et al., 2012).

Herein, we developed a first-ever antibody-drug conjugate (ADC), i.e., anti-CD19 antibody (Inebilizumab)-Volasertib conjugate (V-ADC) via cleavable valine-citrulline (VC) based linker, to increase targeting specificity for B-cell lymphoma cells and to minimize the toxic effects of Volasertib. Among 328 unique antigen targets were approved for antibody-based therapy, the most widely targeted antigen was CD19 (Strohl, 2018). CD19 is a transmembrane glycoprotein widely expressed on normal B cells and a variety of B cell lymphomas/leukemias. Binding of CD19 induces rapid internalization, which is one of the critical features for ADC therapy (Hammer, 2012; Khongorzul, Ling, Khan, Ihsan, & Zhang, 2020). The expected molecular mechanism is that (1) the ADC bounds to the CD19 on the surface of target cells, which rapidly internalized by endocytosis; (2) the complex transports to late endosomes and fuse with lysosomes; (3) the cytotoxic payload is released by linker cleavage and/or antibody degradation within acidic environment and by enzymatic activities (such as cathepsin B) in lysosomes (H. Chen, Lin, Arnst, Miller, & Li, 2017; Kalim et al., 2017). Especially, VC linker was designed to be cleaved by the cysteine protease cathepsin B in lysosomes (Doronina et al., 2003). (4) Finally, the released payload inhibits PLK-1 and induces apoptosis in lymphoma cells.

Apoptosis can be caused in cancer cells by intrinsic and extrinsic pathways depending on the apoptosis-initiating signal. The intrinsic apoptosis is triggered by intrinsic stimuli such as DNA damage, that lead to the activation of transcription factor TP53. TP53 cooperate with other molecules to induce mitochondrial outer membrane permeabilization (MOMP). Subsequently, cytochrome C is released from the mitochondria into cytosol, which is involved in forming of apoptosomes for caspase 9 activation, which further activates caspase 3 and induces Poly (ADP-ribose) polymerase (PARP) cleavage and ultimately cell apoptosis.

In this study, we will elucidate the molecular mechanisms and evaluate the therapeutic efficacy of V-ADC in human B-cell lymphoma cell lines and in xenograft models of aggressive B cell lymphoma.

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

Antibody-drug conjugates (ADC), CD19 antibody-Volasertib conjugate (V-ADC), Polo-like kinase (PLK)-1, G2/M arrest, Apoptosis, TP53, B cell lymphoma

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Original Statement of Works:

Major task #1: Determine PLK-1 mediated activation of oncogenic pathways in lymphoma cells.
- Completed

Major task #2: Determine the significance of PLK-1 in MYC-induced lymphomagenesis.
- Completed.

Major task #3: Determine PLK-1 inhibition confers synthetic lethality to MYC-driven lymphoma.
- Completed.

Major task #4: To develop a practical method for targeting PLK-1 in B-cell lymphoma.
- Ongoing during this period of No-cost extension.

Major task #5: Determine the therapeutic effect of PLK-1 inhibition *in vivo*.
- Ongoing during this period of No-cost extension.

For major task #4: the subtask #1 is to establishment of an antibody-drug-conjugate, we have successfully established the anti-CD19 antibody (Inebilizumab)-Volasertib conjugate (V-ADC), we are in the process of studying the specificity and efficacy of V-ADC in various lymphoma cell lines (Major task #4, subtask #2). For and xenograft animal model of B-cell lymphomas (Major task #5, subtask #2). For major task #5: We are actively working on evaluating the therapeutic effect of PLK-1 inhibition in xenograft and PDX models of aggressive B-cell lymphomas.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

First, we investigated which NHL cell lines were sensitive to Volasertib. A battery of B-NHL cell lines were treated with various dose of volasertib for 48 h and cell viability was determined by PrestoBlue cell proliferation/viability assay (Figure 1 a-d). The study showed that Volasertib inhibited lymphoma cell proliferation in most of the B-NHL cell lines, including mantle cell lymphoma, germinal center B-cell (GCB)-type diffuse large B-cell lymphoma, double hit lymphoma, and Burkitt's lymphoma. Z138, a MCL cell line, was the most sensitive cell line to Volasertib treatment and was selected for further analysis of therapeutic effect of V-ADC. However, our studies showed that V-ADC exhibited only borderline inhibitory effect on lymphoma cell proliferation (Figure 2(a)). We measured the levels of CD19 expression by flow cytometry and found that CD19 expression in Z138 cells was substantially lower than that in other B-NHL cell lines (Figure 2(b)). In addition, we discovered a gene mutant in the exon 3 of CD19 (*P. L174V*) in Z138 cells. We hypothesized that the efficiency of CD19 binding by Inebilizumab could be compromised with low level of CD19 expression as well as with this mutant form, since exon 3 is coding for the extracellular domain of CD19 (Sotillo et al., 2015). Therefore, we engineered stable Z-138 cell lines with overexpression of a wild type CD19 (CD19-WT) and a L174V mutant CD19 (CD19-Mut), respectively, to further study the cytotoxic effect of V-ADC.

We found that both CD19-WT and CD19-Mut stable cell lines expressed significantly higher levels of CD19 comparing to the parental cells (Figure 3 (a)). We then treated these cells with 10nM V-ADC for 48 h and the apoptotic subpopulations was determined by flow cytometry using Annexin V apoptosis detection kit (Figure 3 (b)). We found that cells with CD19-overexpression, both wild type and mutant forms, exhibited markedly increased cell apoptosis comparing to parental cells (53-55% in overexpressing cells versus 5% in parental cells). In addition, V-ADC treatment significantly induced a reduction of mitochondria membrane potential (TMRE), activations of caspase 3 and 9, and cleavage of PARP in both CD19-WT and -Mut Z138 cell lines. We did not identify any significant difference in V-ADC-induced cytotoxic effects between the CD19-WT and -Mut cells. In conclusion, the therapeutic efficacy of V-ADC depends on the levels of CD19 expression and the gene mutation in the exon 3 of CD19 (*P. L174V*) might not have a significant impact in CD19-binding in our system.

For the in vivo examination, we tested V-ADC in the xenograft models of aggressive B-cell lymphoma. Two dosages of intravenous administration of V-ADC per week were well tolerant and with inhibitory effect, although limited, in the experimental mice. Similar to the *in vitro* examinations, CD19 overexpressed xenograft model showed improved response to V-ADC treatment, suggesting that CD19 abundance is an important factor affecting the therapeutic effect. Concurrently, we are actively working on the investigation of improving the therapeutic effects of V-ADC *in vivo*., including identifying efficient combinations and improving the intracellular processing of the V-ADC.

Figure 1

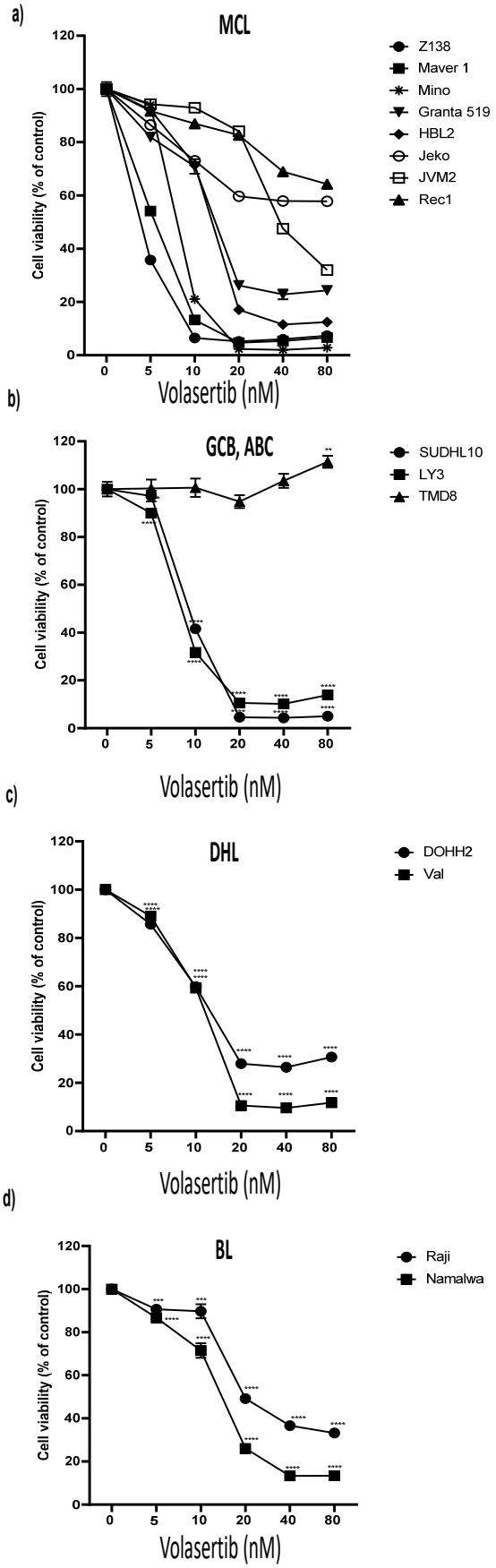


Figure 2

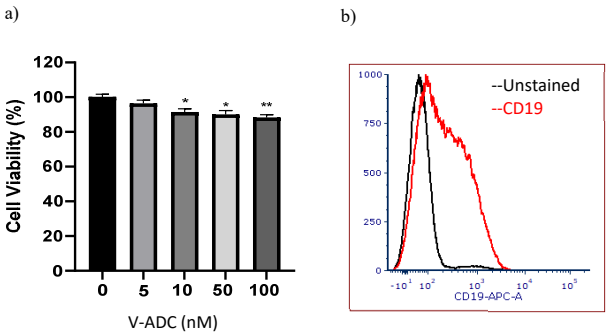
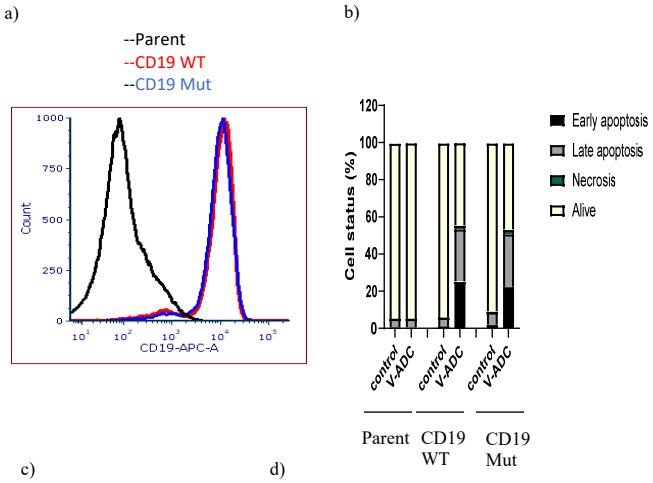
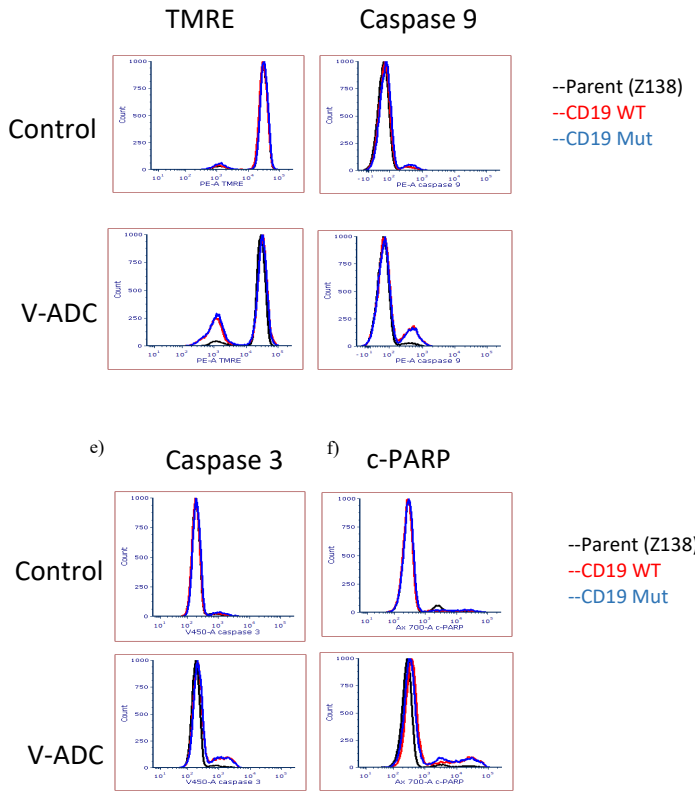


Figure 3





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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The project provided the opportunities for training activities for our post-doctoral fellows and technicians, such as one-on-one work with a mentor to perform cellular and molecular biology assays, like cloning and virus infections, as well as animal studies.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Poster presentation in AACR annual meeting 2023, Abstract #6965
Title: Anti-CD19 antibody-drug conjugate therapy in B cell non-Hodgkin lymphoma
Grand rounds presentations at Roswell Park Cancer Center, Buffalo, New York; City of Hope National Hospital.
Invited speech at 15th Annual Hematology/Hematopathology Symposium. Syracuse, New York

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report. Since we are still working on the completion of animal studies using V-ADC, we may be able to provide additional updates when those data are available.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The findings have a significant impact on putting PLK-1 targeting into real practice, especially in the context of PLK-1 is raising interests in the targeted-therapy development for hematological malignancies. Also, the findings may have a significant impact in future development of clinical trials in which CD19 expression level may affect the therapeutic effect of CD19-mediated ADC therapies/CD19-targeted therapies.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

PLK-1 is considered as a potential therapeutic target in various types of cancers. In the clinical-stage oncology drug candidates database CanSAR and the Therapeutic Target Database, PLK-1 ranks at the top of essential genes for cancer biology. However, as a pan-essential gene, the efficient targeting of PLK-1 with high therapeutic index has been long a dilemma in real clinical practice. The findings of current study suggest that the ADC strategy might be a promising way to address this issue.

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

We do expect the V-ADC will have significant therapeutic efficacy in xenograft/PDX models of aggressive B-cell lymphomas. Therefore, future development of V-ADC could be utilized in future clinical trials.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report.

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We plan to further investigate the therapeutic effect of V-ADC in a PDX model of B-cell lymphoma as proposed in the application. We anticipate that the V-ADC may have potent therapeutic effect in PDX/xenograft models of B-cell lymphoma in vivo. We are actively working on these areas.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

We do not expect any changes on expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

No significant changes

Significant changes in use or care of vertebrate animals

No significant changes

Significant changes in use of biohazards and/or select agents

No significant changes

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Poster presentation in AACR annual meeting 2023, Abstract #6965 Title: Anti-CD19 antibody-drug conjugate therapy in B cell non-Hodgkin lymphoma

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*

- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

No change

Kai Fu, MD, Ph.D.
Dr. Chieko Saito, Ph.D.
Dr. Chengfeng Bi, MD, Ph.D.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

University of Nebraska Medical Center
42nd and Emile St, Omaha, NE 68198

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

N/A

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

N/A