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TITLE: Precision Oncology-Based Therapeutic Targeting in Mesothelioma

PRINCIPAL INVESTIGATOR: Mark Klein

CONTRACTING ORGANIZATION: Center for Veterans Research and Education
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6. AUTHOR(S)

Mark Klein

E-Mail: mark.klein2@va.gov

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1 Veterans Dr., 151
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14. ABSTRACT

Purpose: Given that mesothelioma nearly universally exhibits cell cycle abnormalities and the cell cycle interacts with multiple pathways important to the growth of mesothelioma, disruption of the cell cycle presents as a likely effective approach (or will contribute to a multiple pathway-targeted combination approach) to treating mesothelioma clinically. Our hypothesis is that combined inhibition of 1) multiple cell cycle proteins, and 2) one of 3 separate and alternative molecular pathways will serve as an effective therapy against multiple molecular subtypes of mesothelioma.
Scope: We are utilizing in vitro and in vivo studies to evaluate how to best target molecular subtypes of mesothelioma.
Major Findings: We have determined in multiple cell lines that cell cycle inhibitors (dinaciclib, abemaciclib, palbociclib) and inhibitors of antioxidant defense (gentian violet and auranofin) have significant activity against mesothelioma in vitro.

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:

Mesothelioma is a devastating cancer with a poor prognosis. Defects in cell cycle machinery are the most common molecular feature of mesothelioma tumors. Low expression of the CDK4/CDK6 inhibitor and tumor suppressor p16INK4a has been demonstrated in multiple basic and clinical studies of mesothelioma, affecting up to 90% of all tumors. The cell cycle machinery also interacts with other important targetable pathways within the cell, most notably the 1) PI3K/MTOR pathway, 2) mitochondrial antioxidant defense system, and 3) immune checkpoint system. In this research, we are utilizing in vitro and in vivo studies, as initially informed by genomic and molecular phenotypic information supplied by The NIH Cancer Genome Atlas program, to evaluate how to best target molecular subtypes of mesothelioma.

2. KEYWORDS:

mesothelioma, cyclin-dependent kinase (CDK), cell cycle, mitochondrial antioxidant defense, immune checkpoint, phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (MTOR)

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1. To determine the mechanisms by which cell cycle-specific proteins and interacting major molecular pathways contribute to mesothelioma cell survival and the most optimal cell cycle protein combination to target. (70%)

- **Major Task 1:** Evaluate the mechanisms of CDK4/6 inhibition and prevention or rescue of resistance to CDK4/6 inhibition via CDK2 inhibition by examining the immune system and tumor microenvironment response. Months 1-12. (85%)
- **Major Task 2:** Evaluate whether CDK4/6 inhibition enhances tumor antigen and immune cytokine expression. Months 12-18. (50%)

Specific Aim 2. To evaluate the mechanism by which select major molecular pathways overcome cell cycle inhibition and identify the most effective combination of agents to treat mesothelioma in these molecular subtypes. (0%)

- **Major Task 3:** Determine effects of CDK2 and CDK4/6 inhibitors on T cells in vitro and in vivo. Months 12-18. (10%)
- **Major Task 4:** Determine the efficacy of combined CDK4/6 and immune checkpoint inhibition in a syngeneic mesothelioma xenograft. Months 9-18. (0%)
- **Major Task 5:** Determine the efficacy of combined CDK2 and immune checkpoint inhibition in a syngeneic mesothelioma xenograft. Months 12-18. (0%)

What was accomplished under these goals?

Major activities - Major Tasks 1 and 2
We have dedicated the time predominantly to in vitro experiments in specific aim 1. At the last annual report, we included data on numerous proliferation assays to evaluate in a robust fashion (with experiments conducted in multiple replicates) dinaciclib, auranofin, gentian violet, palbociclib, and abemaciclib. This has included multiple cell lines, including 4 mesothelioma cell lines plus non-malignant cell line Met5A. In addition, we've conducted numerous immunoblot experiments to evaluate protein expression.

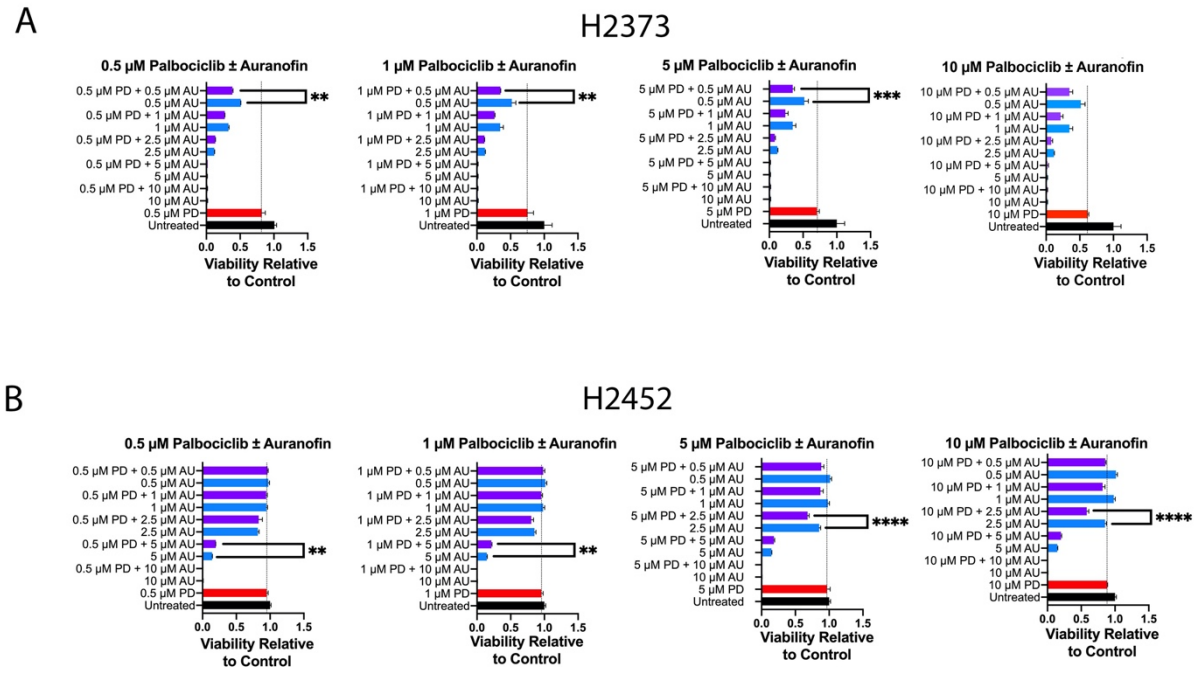
Since the last annual report, we have built on the above. First, we have conducted proliferation experiments with palbociclib plus auranofin to determine if the combination exhibits synergistic, additive, or antagonistic effects on cell proliferation. Second, we have conduced numerous colony forming assays to provide a second, confirmatory, set of experiments to provide evidence for decreased cell proliferation and growth. Third, we have been developing resistance in mesothelial cells to palbociclib, abemaciclib, and dinaciclib. Fourth, we have run numerous immunoblots on cells after experiments with palbociclib, auranofin, dinaciclib, and abemaciclib to determine the effects on protein expression. The following information is not an exhaustive list but a representative example of the data we have obtained in the last year.

Specific Objectives
Specific Aim 1. To determine the mechanisms by which cell cycle-specific proteins and interacting major molecular pathways contribute to mesothelioma cell survival and the most optimal cell cycle protein combination to target.
Specific Aim 2. To evaluate the mechanism by which select major molecular pathways overcome cell cycle inhibition and identify the most effective combination of agents to treat mesothelioma in these molecular subtypes.

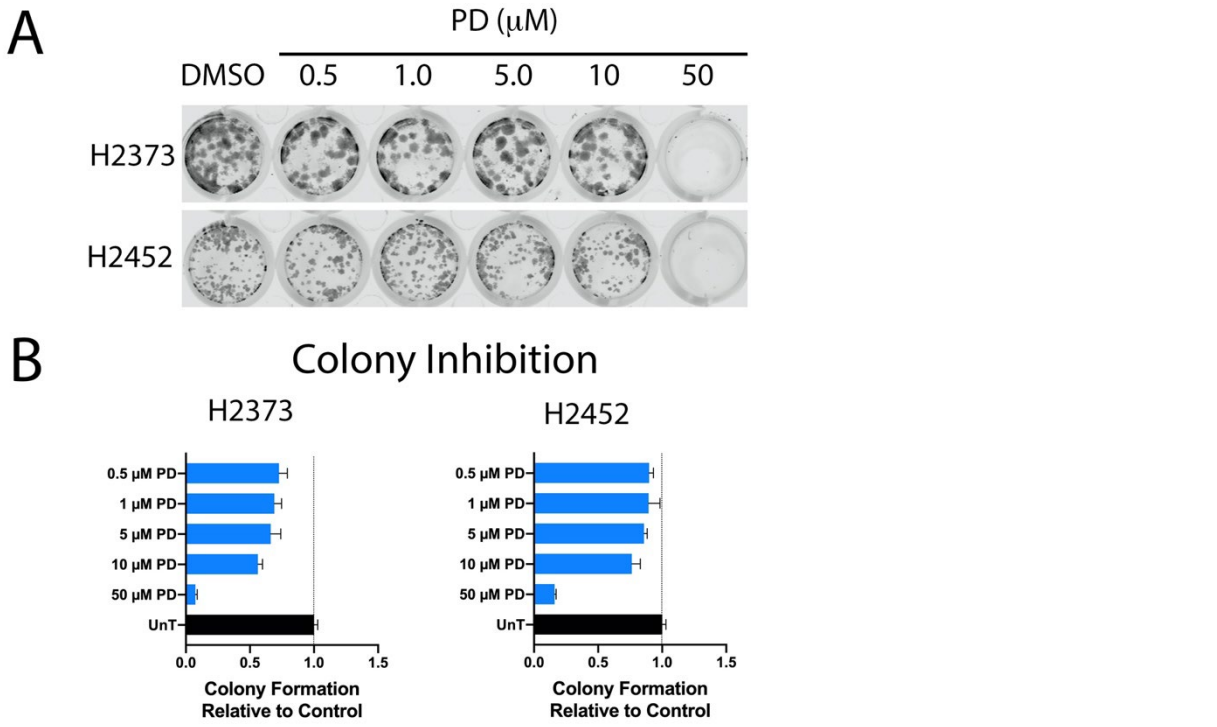
Methodology 1) Proliferation assays in 96 well plates will be performed using a CCK-8 viability assay. At 24-72 hrs after treatment with palbociclib and/or auranofin, cells (H2373 and H2452) were incubated with CCK-8 for 2 h at 37 °C and the absorbance relative to media alone were determined. 2) Colony forming assays were performed in 6 well plates with palbociclib or auranofin in H2373 and H2452 cells. 3) Multiple western blots have been performed on CDKs, cyclins, Trx2, and related proteins after treatment with palbociclib or auranofin. 4) Palocilcib and auranofin (other drugs currently under evaluation) at the IC_{50} concentration were added to cells, and 24-72 h post–treatment the % of the cells in G_0/G_1, S, and G_2 were determined by propidium iodide (PI) staining and FACS analysis to determine the effects on the cell cycle. 5) A xenograft experiment was conducted with H2373 cells.

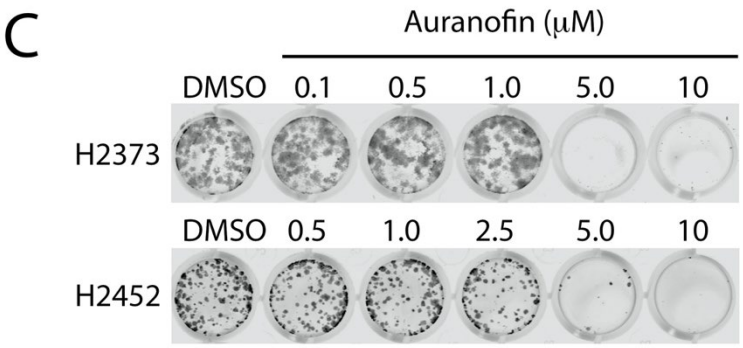
Significant results/key outcomes

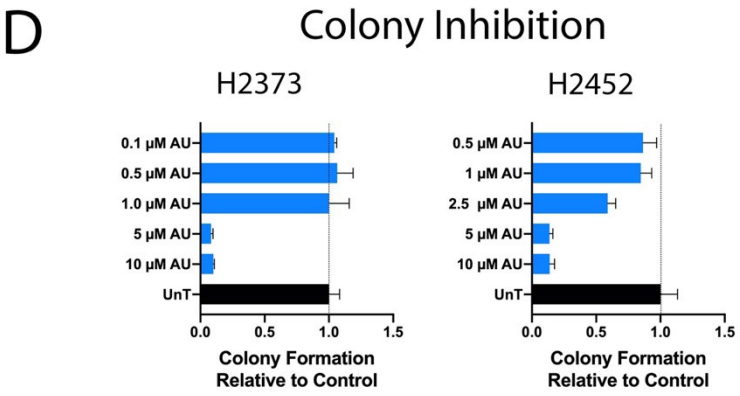
First, as shown in the figure below, we determined that for several concentrations of palbociclib and auranofin, the combination was synergistic against mesothelioma cell grown in cell lines H2373 and H2452.



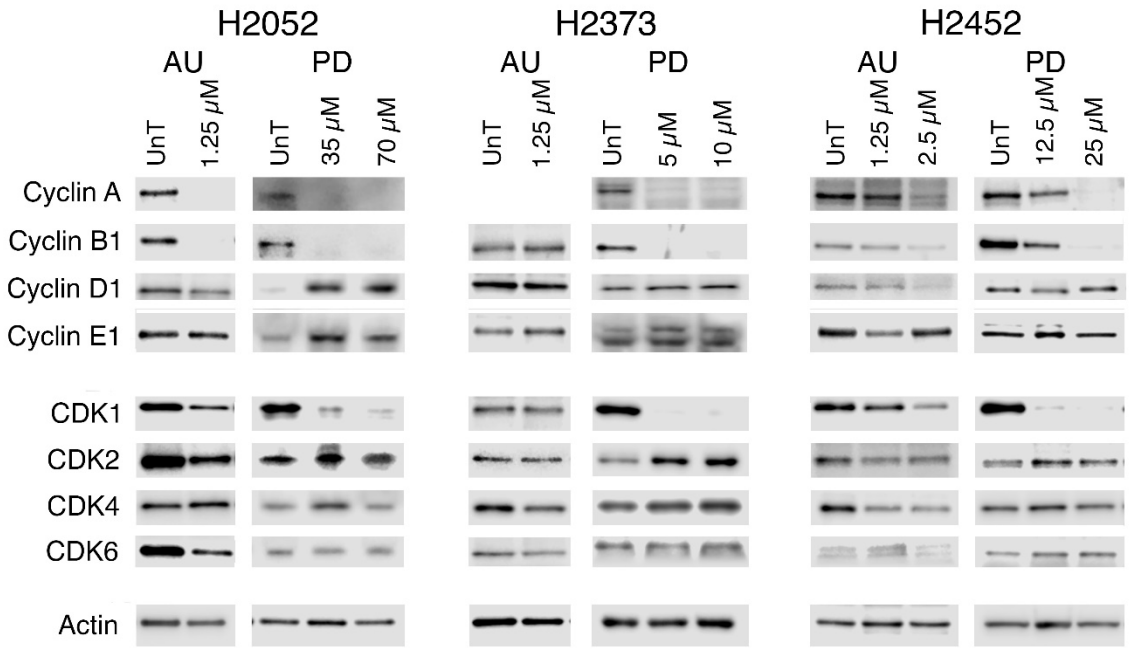
Second, the figures below demonstrate that colony formation is decreased after treatment with palbociclib or auranofin.



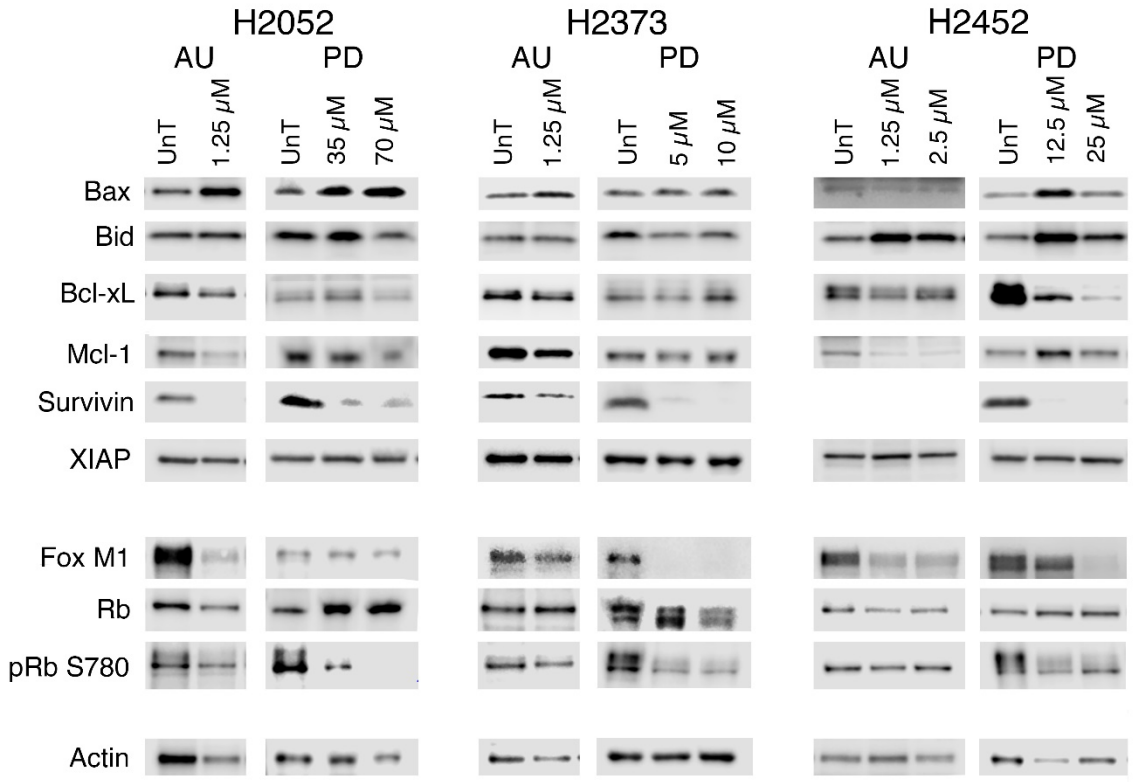




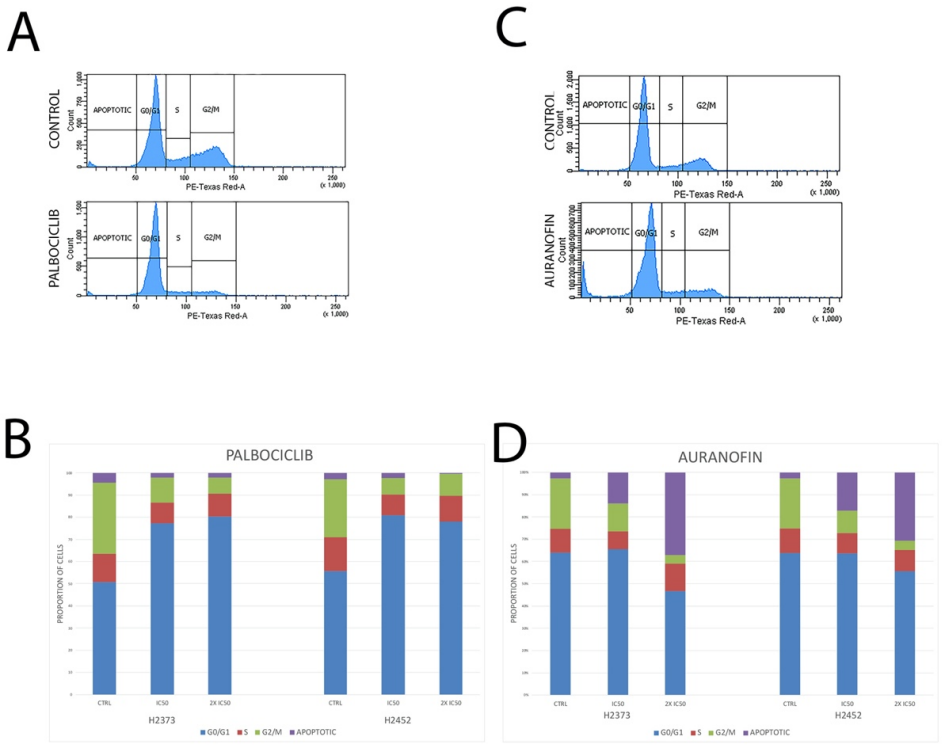
After treatment with palbocilcib or auranofin, significant downregulation occurred for multiple cyclins and CDKs.



After treatment with palbocilcib or auranofin, in addition the above, significant effect on apoptosis-related proteins were observed.
Cell



Cell cycle was significantly disrupted after treatment with palbociclib or auranofin in H2373 cells.



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

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report.

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

We will conduct our animal experiments later this calendar year and in early 2022. In addition, we will complete the rest of the proposed in vitro assays on the PI3K/MTOR pathway and the in vitro T cell experiments. With the no-cost extension, we will be able to

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We confirmed that palbociclib and auranofin are synergistic against mesothelioma growth in vitro. As a result, we will report this to our VA Technology Transfer Office to discuss whether they would proceed with a New Use patent.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Previously we thought there was nothing to report. However, after discussion with the VA Office of Technology Transfer, we were informed that the results obtained in this project should be reported to their office for consideration of a “new use” patent, and we are in the midst of preparing this report. No other technology transfer activities have taken place.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Due to COVID-19 induced delays, we requested a no-cost extension that was granted.

Actual or anticipated problems or delays and actions or plans to resolve them

COVID-19 delays from the previous reporting period pushed much of the work to the last reporting period (ending 9/14/2020). With the second COVID-19 surge, Dr. Klein was requested to telework for a couple months. The rest of the laboratory personnel were allowed to stay on site. Once of the lab members had to take a medical leave for several weeks.

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

Significant changes in use or care of human subjects

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.

Nothing to report. We submitted to Antioxidants, but they felt it was not closely enough aligned with their journal goals. We are editing the manuscript for submission to Neoplasia.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to Report.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Previously we thought there was nothing to report. However, after discussion with the VA Office of Technology Transfer, we were informed that the results obtained in this project should be reported to their office for consideration of a “new use” patent, and we are in the midst of preparing this report. No other technology transfer activities have taken place.

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Mark Klein
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Klein is the PI on the project.
Funding Support:	The PI salary is provided by the Minneapolis VA Healthcare System.

Name:	Betsy Kren
Project Role:	co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Kren is a co-investigator. She works closely on animal experiments and immunblotting with Steph Porter. She also conducts the RT-PCR experiments.
Funding Support:	All effort on this project is supported by this DOD award.
Name:	George Scaria
Project Role:	Biologic Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Dr. Scaria aids in animal experiments and conducts multiple in vitro experiments, including proliferation assays, flow cytometry, and apoptosis assays. He will be conducting the T-cell work.
Funding Support:	All effort on this project is supported by this DOD award.
Name:	George Scaria
Project Role:	Biologic Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Dr. Scaria aids in animal experiments and conducts multiple in vitro experiments, including proliferation assays, flow cytometry, and apoptosis assays. He will be conducting the T-cell work.
Funding Support:	All effort on this project is supported by this DOD award.
Name:	Marian Kratzke
Project Role:	Biologic Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Kratzke predominantly conducts in vitro experiments, including a bulk of the proliferation assays. She also prepares cells for animal experiments.
Funding Support:	All effort on this project is supported by this DOD award.
Name:	Stephan Porter
Project Role:	Biologic Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Kratzke predominantly conducts in vitro experiments, including a bulk of the proliferation assays.

Name:	Khalil Ahmed
Project Role:	co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6
Contribution to Project:	Dr. Ahmed is a co-I on the project and provides regular input into experimental design and interpretation.
Funding Support:	The co-I salary is provided by the Minneapolis VA Healthcare System.
Name:	Robert Kratzke
Project Role:	co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6
Contribution to Project:	Dr. Kratzke is a co-I on the project and provides regular input into experimental design and interpretation. His lab also provides cell lines and experimental expertise as available.
Funding Support:	The co-I salary is provided by the University of Minnesota.

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

The following 2 grants are new for Dr. Klein as PI. There is no scientific or budgetary overlap for each with the current DOD Award.

Veterans Affairs Cooperative Studies Program
Lung Cancer Precision Oncology Program (LPOP)
Title: Upper Midwest VA Lung Cancer Screening and Research Network
PI: Mark Klein, M.D. (co-PIs: Christine Wendt, M.D. and Apar Ganti, M.D.)
Percent Effort: 4.5 calendar months
Amount: total over 5 years
Dates: 3/08/2021-03/07/2026

Randy Shaver Cancer Research and Community Fund
Grant
Title: Identification of Novel Drug Combinations for the Treatment of Mesothelioma
PI: Mark Klein, M.D.
Percent Effort: 0.6 calendar months
Amount:
Dates: 02/01/2021-01/30/2022

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: