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TITLE: Targeting High Mobility Group Box Protein 3 to Sensitize Chemoresistant Human Ovarian Cancer Cells to Cisplatin In Vivo

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CONTRACTING ORGANIZATION: The University of Texas, Austin, TX

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14. ABSTRACT

The 5-year survival rate for ovarian cancer patients in the United States is only ~48%. This dismal survival rate is largely due to high rates of cancer relapse, recurrence, and development of chemoresistance. Many chemotherapeutic drugs used to treat ovarian cancer induce DNA damage, and the efficient removal of such drug-induced DNA damage is one of the mechanisms that contributes to chemoresistance. We have recently demonstrated that cisplatin-resistant human ovarian cancer cells can be sensitized to cisplatin by targeting the non-histone architectural protein, High Mobility Group Box 3 (HMGB3). HMGB3, unlike its family members, HMGB1 and HMGB2, is only expressed in highly dividing cells, and is over-expressed in many ovarian tumors, such that HMGB3 may provide a selective target for cancer therapy. We have shown that the DNA damage response (DDR) kinases ATR, CHK1, and ATM are significantly down-regulated at the mRNA level, as a function of HMGB3 in human ovarian cancer cells. Further, we found that HMGB3 can localize to cisplatin-induced DNA lesions to assist in their repair, such that its depletion can significantly reduce the removal of cisplatin-DNA adducts from chemoresistant human ovarian cancer cells, leading to increased cytotoxicity and apoptosis. These results suggest that HMGB3 is a promising novel target for ovarian cancer therapy. We propose in this pilot project to assess the effects of targeting HMGB3 in vivo to sensitize chemoresistant human ovarian tumors to cisplatin treatment.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

Ovarian cancer is the fifth most common cause of cancer-related death in women in the United States. First-line chemotherapy includes DNA-damaging drugs such as cisplatin. Unfortunately, the 5-year survival rate is only ~48%, and the cancer recurrence rate is ~70%. Drug resistance contributes to cancer recurrence and poor patient survival. If tumor cells can repair and remove anticancer drug-DNA lesions, then cancer drug resistance can develop. Thus, improved treatment strategies are sorely needed. There is a critical need to identify novel drug targets to treat patients who have stopped responding to chemotherapy (i.e., chemoresistant patients). Our goal is to study the effects of inhibiting a novel target, the High Mobility Group Box 3 (HMGB3) protein, to sensitize chemoresistant ovarian tumors to the chemotherapeutic drug, cisplatin. We found that HMGB3 participates in the repair of chemotherapeutic drug-induced DNA lesions; thus, by depleting this protein we can restore killing of formerly drug-resistant human ovarian cancer cells. This pilot project aims to identify small molecule inhibitors of HMGB3 to study the effects of HMGB3 inhibition on tumor growth and the sensitization of chemoresistant human ovarian cancer cells to cisplatin treatment in vivo. Successful targeting of HMGB3 in a tumor microenvironment may open new therapeutic options for patients suffering from chemoresistant and recurrent forms of ovarian cancer to improve the quality of life and overall survival of these patients.

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

Ovarian cancer, chemoresistance, HMGB3, DNA damage repair, DNA damage response, HMGB3 as novel target, sensitization of chemoresistance

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Goal 1: Identify small molecules that interfere with HMGB3 binding to DNA and enhance cytotoxicity in ovarian cancer cells. The project timeline was 1-12 months. Approximately 50% of the project has been completed.

Major Goal 2: Determine the effect of HMGB3 silencing using the inducible shRNA method in sensitizing chemoresistant human ovarian tumor xenografts to cisplatin treatment in mice. The project timeline was 1-6 months. Approximately, 50% of the project has been completed.

What was accomplished under these goals?

Major Goal 1:

Major activities

Binding assays were performed using purified HMGB3 protein and purified DNA triplex substrates, demonstrating that HMGB3 binds to a 57 base-pair DNA duplex substrate and to a triplex-forming oligonucleotide (TFO) targeted DNA interstrand crosslink (ICL) containing triplex DNA substrate.

Further, small molecules identified from a previous virtual screening were used in these binding studies to determine if these molecules could alter the HMGB3 and DNA (both duplex and ICL containing triplex DNA substrates) complex formation. We were able to identify a molecule that inhibited the binding of HMGB3 to the DNA duplex substrate. These results indicate that this method can be used to efficiently identify small molecules that can inhibit HMGB3's ability to bind DNA, resulting in identification of small molecule inhibitors which can be used in future preclinical studies.

Specific objectives

a. Determine whether HMGB3 binds to double-stranded DNA and to TFO-directed ICL-containing triplex DNA with high affinity, forming stable complexes *in vitro*.
b. Establish this binding assay as a valid experimental approach to identify small molecules that can disrupt the binding of HMGB3 to DNA substrates.
c. Develop an assay to screen for HMGB3 inhibitors to be used in the proposed studies.

Significant results

In vitro binding assays demonstrated that HMGB3 binds to a radiolabeled 57 base-pair duplex DNA substrate with high affinity (Figure 1) and forms distinct complexes. In addition, HMGB3 binds to TFO-directed ICL-containing triplex DNA substrates with high affinity and forms distinct complexes.

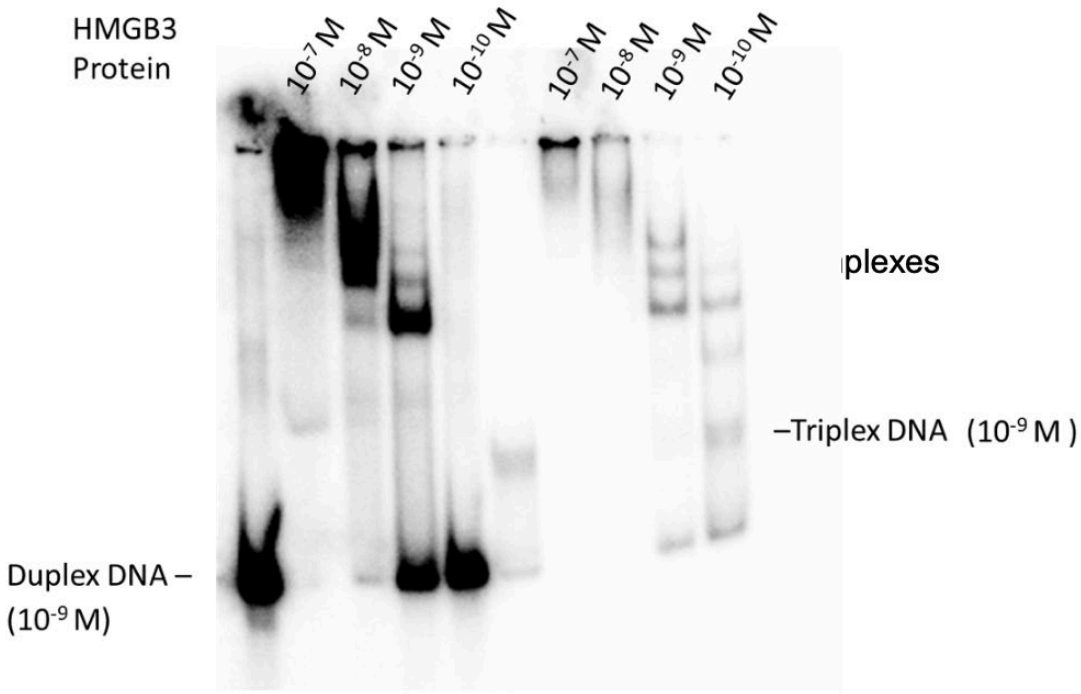

Figure 1: HMGB3 binds to 57 bp duplex DNA and TFO-directed ICL containing triplex DNA.

Goals not met

High through-put screening using a commercially available library of approximately 80,000 small molecules is still a work in progress. The high throughput buffer and other conditions are being optimized currently to ensure effective DNA-protein complex formation.

Description of the methodology

A 57 base pair duplex DNA substrate was radiolabeled using γ32P-ATP. For *in vitro* DNA-protein binding assays, 10^{-9} M radiolabeled duplex DNA was incubated with 10^{-7} – 10^{-10} M purified HMGB3 protein in 1X binding buffer for 20 minutes at 30°C in a 20 µL total reaction volume. The complexes were resolved by loading them on a 5% polyacrylamide gel and running them for 2 hours at 4°C using 1X TBE buffer. Gels were then dried and exposed overnight on a phosphor-screen. Complexes were visualized using Typhoon scanner. To prepare the triplex DNA substrate, equimolar amounts of 5'-psoralen containing 30-mer oligos were incubated with 57 bp radiolabeled duplex DNA overnight at 37°C in 1X triplex binding buffer. Subsequently, the triplexes were irradiated with 1.8 J/cm2 UVA to induce DNA interstrand crosslinks. These TFO-ICL substrates were then used for *in vitro* binding assays to determine HMGB3- triplex complex formation as mentioned above.

Major Goal 2:

Major activities

The institutional animal protocol for this project has been submitted to the University of Texas IACUC committee for a 3-year renewal.

Chemosensitive A2780 and chemoresistant A2780/CP70 ovarian cancer cells were transfected with Tetracycline repressor (Tet R) expressing plasmid pcDNA6/TR (purchased from Invitrogen) containing blasticidin selection genes. Both the cell lines were selected with 10 µg/ml blasticidin for 21 days. Two tetracycline repressor-expressing chemosensitive ovarian cancer cell lines (A2780 TetR #1 and A2780 TetR #2) and two chemoresistant A2780/CP70 cell lines (CP70 TetR #1 and CP70 TetR #2) were developed. HMGB3 shRNA sequences were designed using the Invitrogen siRNA tools and two shRNAs were designed against HMGB3 transcript variants 2 and 4. ShRNA oligos were purchased from IDT and were cloned into the pENTR/THT plasmid vector which contains an H1 promoter that is regulated by tetracycline. However, both pcDNA6/TR and pENTR/THT vectors were indicated to use blasticidin as the selection antibiotic. It was determined that using the same antibiotic for selection of both vectors would significantly interfere with identifying cells with both vectors. The HMGB3 shRNA expression vector contains a kanamycin resistance gene (and thereby potentially G418 resistance for selection in mammalian cells). A2780 cells were transfected with HMGB3 shRNA plasmid and are being treated with either blasticidin or G418 to confirm resistance to these selection antibiotics.

A novel interaction between cisplatin resistance associated overexpressed protein (CROP/LUC7L3) and HMGB3 was verified, and the role of LUC7L3 in sensitizing cisplatin resistant ovarian cancer cells was studied using siRNA-based methods.

Specific objectives

a. Generate tetracycline (or doxycycline) inducible HMGB3 shRNA expressing chemosensitive A2780 and chemoresistant A2780/CP70 ovarian cancer cells lines. These cell lines will be used to develop mouse xenografts to test the effects of HMGB3 inhibition on tumor growth.

Significant results

Chemosensitive A2780 and chemoresistant A2780/CP70 human ovarian cancer cells lines have been successfully transfected with a TetR expressing pcDNA6/TR plasmid containing a blasticidin selection marker. Two tetracycline repressor-expressing chemosensitive ovarian cancer cell lines (A2780 TetR #1 and A2780 TetR #2) and two chemoresistant A2780/CP70 cell lines (CP70 TetR #1 and CP70 TetR #2) were constructed (Figure 2).

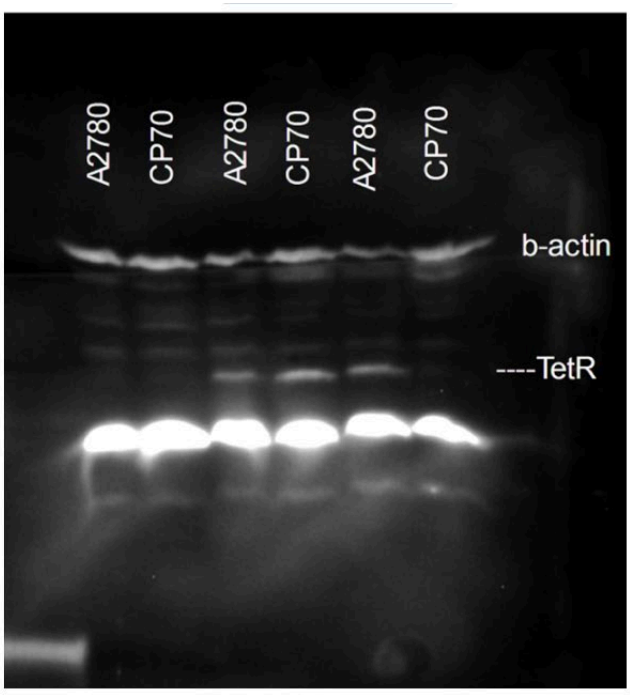

Figure 2: Chemosensitive A2780 and chemoresistant A2780/CP70 cells expressing tetracycline repressor (TetR) protein.

Four HMGB3 transcript variants were identified. Not all 4 transcript variants were targetable. We found that transcript variants 2 and 4 were targetable. HMGB3 shRNAs were successfully cloned into the tetracycline inducible pENTR/THT vectors. A2780 cells were transiently transfected with pENTR/THT + HMGB3 shRNA plasmid and are being treated with either blasticidin or G418 to confirm resistance to these selection antibiotics. Preliminary results indicate that the pENTR/THT+HMGB3 shRNA plasmid confers resistance to G418 but not blasticidin (Figure 3).

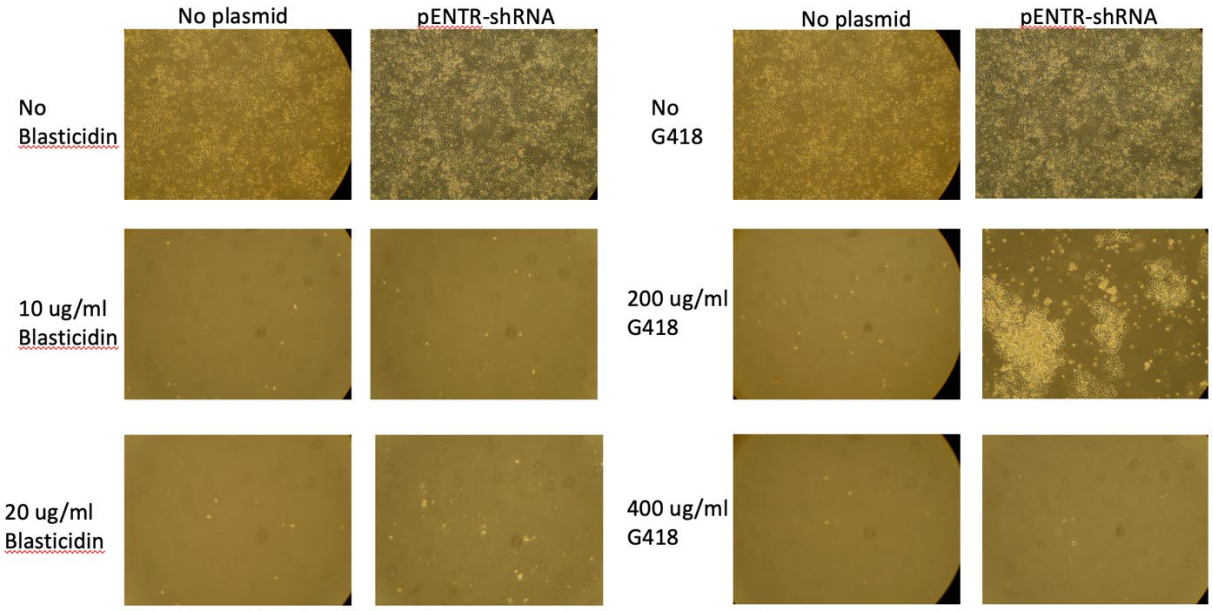

Figure 3. Photo of A2780 cells following transfection with pENTR/THT+HMGB3 shRNA plasmid and treatment with blasticidin or G418 for 21 days.

Using mass spectrometry, we have previously observed that HMG3 interacted with cisplatin resistance associated overexpressed protein CROP, also known as LUC7L3 protein, which is a core factor of the spliceosome. We studied the role of targeting LUC7L3 in both A2780 and chemoresistant A2780/CP70 cells. In the chemosensitive A2780 cells, siRNA mediated depletion of LUC7L3 was lethal (Figure 4). However, depleting LUC7L3 in chemosensitive A2780/CP70 ovarian cancer cells did not show severe lethality when compared to A2780. This may indicate an important physiological difference in between chemosensitive and chemoresistance human ovarian cancer cells. However, HMGB3 being a transcription modulator of DNA damage response (DDR) kinases ATR and CHK1, its association with a spliceosome core factor, LUC7L3, may indicate a possible mechanism of how HMGB3 may be connected to transcription modulation of DDR kinases in the chemoresistant cells.

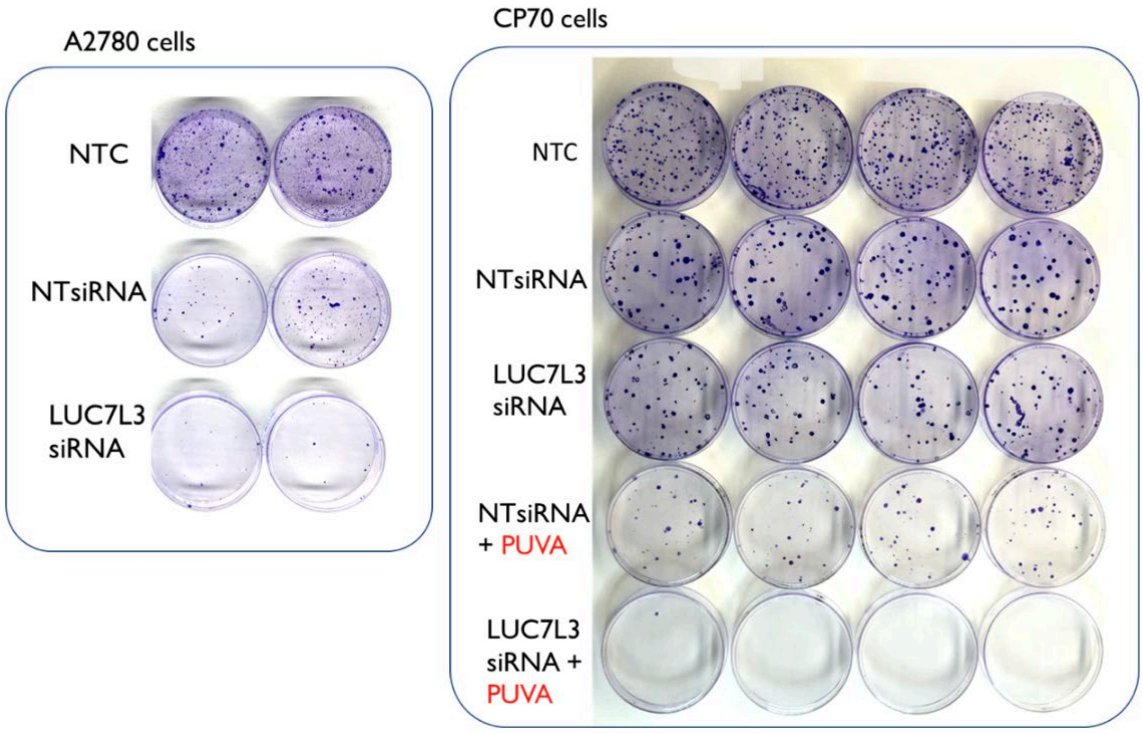

Figure 4: SiRNA-mediated depletion of LUC7L3 sensitizes chemosensitive A2780 and chemoresistant A2780/CP70 cells and may offer an explanation on how HMGB3 modulates the expression levels of DNA damage response kinases.

Description of methodology

A2780 and A2780/CP70 human ovarian cancer cells were grown and maintained in RPMI media with 1% penicillin/streptomycin and 10% bovine serum albumin (BSA) with 5% CO2 at 37°C. Two µg of tetracycline repressor (TetR) expression vector pcDNA6/TR was transfected into A2780 and A2780/CP70 cells using Geneporter transfection reagent using the manufacturer's protocol. Transfected cells were further selected with 10 µg/mL blasticidin (Invitrogen) for 21 days to select for TetR expressing cells. ShRNA sequences to target HMGB3 mRNA were designed using the ThermoFisher BLOCK-iT siRNA designer online tool. To express the shRNA, the plasmid pENTR/THT with a H1 promoter was obtained from Addgene. For directional cloning of the HMGB3 shRNA sequences into the pENTR/THT plasmid, the plasmid was digested using BglII and HinDIII restriction enzymes. The double digested product was gel purified and treated with CIP (Calf Intestinal Phosphatase, BIORAD). The shRNA oligos targeting HMGB3 transcript variants 2 and 4 were purchased from IDT with 5′ and 3′ end modifications for directional cloning. The oligos were then treated with T4 PNK (polynucleotide kinase) to add a phosphate group. The oligos and the double digested vectors were then phenol:chloroform:isoamyl alcohol extracted, ethanol precipitated and resuspended in 20 µL nuclease-free water. Ligation was performed in a 50 µL reaction volume using T4 DNA ligase following the manufacturer's recommended protocol. The ligated products were purified. Electrocompetent *E. coli* MBM7070 cells were transformed with the purified ligated

plasmids containing either transcript variant 2 or transcript variant 4, and colonies were selected on kanamycin-agar plates. The shRNA construct was purified from overnight culture of single colony. Five μg of HMGB3 shRNA variant 4 expression vector was transfected into human A2780 cells using the Geneporter transfection reagent. Transfected cells are being selected with either blasticidin or G418 to verify resistance to these two selection antibiotics.

Goals not met

Chemosensitive A2780 and chemoresistant A2780/CP70 xenografts with an inducible HMGB3 shRNA system were planned to be established in nude mice during this time frame. However, the BLOCK-iT kit that we had originally proposed to use from ThermoFisher got discontinued and we had to design another tetracycline (or doxycycline) inducible vector system. Additionally, the development of chemosensitive A2780 and chemo-resistant A2780/CP70 cells with a tetracycline (or doxycycline) inducible HMGB3 shRNA system has been delayed due to concerns regarding the use of blasticidin as the selection antibiotic for both vectors. Experiments are underway to determine the feasibility of using G418 as a second antibiotic.

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

Preliminary data were presented in poster session in the American Association of Cancer Research (AACR) 2022 conference that took place in New Orleans, LA.

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?

1) Transiently transfect A2780 and A2780/CP70 cells with HMGB3 shRNA expression vectors to ensure that the shRNA constructs are efficiently depleting HMGB3 after induction with doxycycline.
2) The most efficient construct will be further transfected in TetR expressing A2780 and A2780/CP70 cells and will be further selected with blasticidin for 21 days. These selected cells will be further checked for HMGB3 depletion upon treatment with doxycycline.
3) Use the cell lines mentioned in 2 to establish tumors in nude mice and test the efficacy of chemotherapy as a function of HMGB3 depletion in chemosensitive and chemoresistant ovarian cancer cells.
4) Study DNA damage response in the xenograft-derived tumor tissues.
5) High throughput screening to identify small molecule inhibitors of HMGB3-DNA interactions.
6) Use an HMGB3 inhibitor in xenograft-derived tumors to assess efficacy of chemotherapy in chemosensitive and chemoresistant tumors

4. **IMPACT:**

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

Ovarian cancer is the most lethal gynecological malignancy and is the fifth most common cause of cancer-related death in women. The devastating consequences of ovarian cancer are primarily due to the lack of early detection and unimproved therapeutic approaches. The mainstay of ovarian cancer treatment is surgical removal of the primary tumor followed by combination chemotherapy. Unfortunately, the 5-year survival rate for these patients is only ~50%, and the disease recurrence rate is devastating at ~70%. This is often due to the development of chemoresistance. Thus, the current therapeutic approaches do not meet the pressing need for efficacious treatments for chemoresistant and recurrent forms of ovarian cancer. One of the molecular processes that significantly contributes to the development of chemoresistance and recurrence in ovarian cancer is the efficient removal of chemotherapeutic DNA damage (such as cisplatin-DNA adducts) from cancer genomes. Therefore, targeting the DNA damage repair system in these chemoresistant cancer cells presents a substantial opportunity to create an impact and advance the field of ovarian cancer research, with the long-term goal of improving the quality of life and the lifespan of these patients. Toward this goal, we have shown that silencing of the architectural non-histone High Mobility Group Box 3 (HMGB3) protein sensitized chemoresistant human ovarian cancer cells to cisplatin. By targeting HMGB3 in tumors in vivo we propose to substantially impact the outcome of chemotherapy by increasing the efficacy of treatment, particularly for those patients who have developed chemoresistant forms of ovarian cancer. In this regard, HMGB3 is a promising target in that, unlike the other HMGB family members, it is highly expressed only in actively proliferating cells, such as cancer cells, and thereby may provide a selective target for ovarian cancer therapy. Thus, the identification and characterization of small molecule inhibitors of HMGB3 to sensitize chemoresistant human ovarian tumors to chemotherapy in vivo is the focus of this pilot study.

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?

There are ~200,000 active-duty female service members and ~1.9 million family members of the active-duty service members in the military. Approximately 14,000 Americans die from ovarian cancer each year. A challenge to date is the management of the high occurrence of chemoresistance and recurrence, which often lead to death from ovarian cancer. Our proposed studies directly support the OCRP research priorities by 1) developing and validating new models to understand recurrence of ovarian cancer, and 2) investigating tumor and host responses to therapy, including tumor survival, cell death, and drug resistance. In addition, more than 600,000 Americans are predicted to die in 2020 from all cancers combined, often due to chemoresistant and recurrent forms of cancers. Therefore, targeting HMGB3, as we have proposed, is not only relevant to the mission of the OCRP to prevent,

treat, and cure ovarian cancer, but it may also open up a novel strategy to confer clinical benefit for the chemoresistant and recurrent forms of many other types of cancer, which will improve the quality of life and increase the lifespan of cancer patients in the United States.

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

The goal of Specific Aim 1 was to screen libraries to identify small molecules that can inhibit the DNA binding function of HMGB3. Our interactions with the drug screening core facility have been slowed due to challenges in developing the assay for HMGB3 binding in a high throughput format. This was also due in part to the COVID pandemic, since the core facility was short staffed during the first year of the project and is still not functioning at 100%. We are working to improve the assay for use in a high throughput screen.

Dr. Anirban Mukherjee, a Research Associate working on the project, left the lab for a career opportunity in industry. It took additional time to find an adequate replacement for his effort on the project with the appropriate skills and credentials to aid in the research. We have now secured this position, but this has slowed progress on both Aims 1 and 2 of the project. We are now in a position to make progress on the project in an effort to reach our projected milestones, given a one-year extension.

Changes that had a significant impact on expenditures

Dr. Anirban Mukherjee, a Research Associate working on the project, left the lab for a career opportunity in industry. It took additional time to find an adequate replacement for his effort on the project with the appropriate skills and credentials to aid in the research. We have now secured this position, but this has slowed progress on both Aims 1 and 2 of the project. We are now in a position to make progress on the project in an effort to reach our projected milestones, given a one-year extension.

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

Nothing to report.

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.

  Nothing to report.

  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**

  Nothing to report.

- **Other Products**

  Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Karen Vasquez
Project Role:	PI
Researcher Identifier (NIH Commons ID):	KVASQUEZ
Nearest person month worked:	1
Contribution to Project:	Dr. Vasquez, the PI, directed the experiments to be performed, provided advice to advance the project. and managed budgets (with Ms. Laura Christensen) and progress reports.
Funding support:	Nothing to report

Name:	Laura Christensen
Project Role:	Research Associate
Researcher Identifier (NIH Commons ID):	LACHRISTENSEN
Nearest person month worked:	2
Contribution to Project:	Wrote animal use protocol for 3-year renewal. Performing experiments to develop chemosensitive A2780 and chemoresistant A2780/CP70 cells with tetracycline (doxycycline) inducible HMGB3 system.
Funding support:	Nothing to report

Name:	Jill Dangerfield
Project Role:	Graduate student
Researcher Identifier (NIH Commons ID)	JILLGERBERICH
Nearest person month worked:	4
Contribution to Project:	Jill took over for Dr. Mukherjee who left the project for another job opportunity. She has kept in contact with him to continue the studies, and compile the data that he had generated.
Funding support:	Nothing to report

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:

Nothing to Report

QUAD CHARTS:

Nothing to Report

9. APPENDICES:

Nothing to Report