AWARD NUMBER: W81XWH-21-1-0392

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

PRINCIPAL INVESTIGATOR: Karen Vasquez

CONTRACTING ORGANIZATION: The University of Texas, Austin, TX

REPORT DATE: June 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2022	Annual	15May2021-14May2022

4. TITLE AND SUBTITLE

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

5a. CONTRACT NUMBER
W81XWH-21-1-0392

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Anirban Mukherjee and Karen Vasquez

E-Mail: karen.vasquez@austin.utexas.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas at Austin, College of Pharmacy

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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 depletion 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 chemotherapy. 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.

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
Unclassified	Unclassified	Unclassified	Unclassified	13	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION

Mortality rates of ovarian cancer patients have declined by ~1% to 2% per year over the past few decades; however, the 5-year survival rate for ovarian cancer patients is dismal at ~48% in the United States. The mainstay of treatment is surgical resection followed by chemotherapy. Unfortunately, ~70% of patients treated with chemotherapy will suffer a relapse. The prognosis of ovarian cancer patients following relapse is very poor and ~50% of these patients will die within 2 years due to the development of chemoresistance and recurrence. Thus, the high frequency of ovarian cancer relapse and the development of chemoresistance and recurrence present significant challenges that must be addressed with high priority to improve the quality of life and the life expectancy for these patients. Many chemotherapeutic drugs used to treat ovarian cancer patients induce DNA damage as part of their mechanism of action. The chemotherapeutic regimens used in the clinic for patients who experience a relapse within 6 months of first-line treatment can include paclitaxel/topotecan/doxorubicin, and patients who experience relapse after 6 months are often treated with platinum-based chemotherapy with or without bevacizumab or Olaparib. These chemoresistant cancer cells often have a high capacity to repair chemotherapeutic drug-induced DNA damage and can effectively efflux the drugs out of the cells, both of which contribute to chemoresistance. Therefore, intervening with the DNA damage repair capacity of chemoresistant ovarian cancer cells is a promising approach to sensitize these cells to further chemotherapeutic treatment. A database search from Clinicaltrials.gov revealed that 2 of 162 ongoing clinical trials worldwide on the treatment of chemoresistant ovarian cancers include the modulation of DNA damage responses (DDR) via inhibition of the checkpoint kinases, CHK1 and CHK2, to prevent the efficient removal of chemotherapy-induced DNA adducts. Thus, modulating DNA repair efficiency to overcome chemoresistance, as proposed in this pilot project, has clinical potential.

Chemotherapeutic drugs (e.g. cisplatin, carboplatin, oxaliplatin; NOTE: in this study we will use cisplatin as the DNA-damaging agent for comparison to our previous results. However, we will also consider the use of carboplatin and oxaliplatin in future studies for clinical relevance) can induce damage and distortions in DNA that are recognized by DNA repair and architectural proteins for subsequent processing. The chromatin-associated non-histone High Mobility Group Box (HMGB) proteins bind to such distorted/damaged DNA with high affinity. The HMGB protein family consists of 4 members that support DNA-dependent processes, such as replication, recombination, transcription, and DNA damage repair. One family member, HMGB3, in contrast with the other members, is overexpressed in highly proliferating cells, such as cancer cells, compared to normal cells, and thus may present a selective target for the treatment of cancer. Although little is known about the role(s) of HMGB3 in chemotherapeutic DNA damage processing, targeting HMGB3 in a mouse model of human breast cancer showed inhibition of cell proliferation and tumor growth through the interaction of HMGB3 with hypoxia-inducible factor 1α. This finding suggests that HMGB3 has the capacity to mitigate cancer development and progression in vivo. We have recently demonstrated that HMGB3 depletion (via siRNA) sensitized cisplatin-resistant human ovarian cancer cells to lower doses of cisplatin. This was achieved in part, as a result of decreased transcription of the DDR kinases, ATR and CHK1, which led to increased apoptosis of the chemoresistant human ovarian cancer cells. Consistent with these findings, we have observed that N-terminal GFP-tagged HMGB3 formed foci in human ovarian cancer cells following cisplatin treatment, suggesting that HMGB3 was recruited to the cisplatin-induced DNA damage in the nucleus. Additionally, we found that the depletion of HMGB3 in cisplatin-resistant A2780/CP70 human ovarian cancer cells reduced the efficiency of cisplatin-DNA adduct removal, likely contributing to the increased apoptosis in these cells. In a different human cell line, U2OS osteosarcoma cells, we observed increased chromosomal aberrations as a function of transient HMGB3 depletion following DNA interstrand crosslink (ICL) induction by treatment with psoralen and UVA irradiation (at 365 nm). This observation suggested that HMGB3 depletion resulted in an increased level of genomic instability while processing chemotherapeutic DNA lesions, consistent with our previous results showing increased apoptosis in chemoresistant ovarian cancer cells following treatment with cisplatin. The ability of HMGB3 to modulate drug-induced DNA damage processing, and its overexpression in cancer cells makes it a promising target to sensitize chemoresistant forms of cancer to chemotherapy. In addition to ovarian cancer, HMGB3 is overexpressed in other cancer cell types compared to normal cells and it has been associated with cancer patient outcomes. Thus, 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

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?**

    1) **Major Task 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.

    2) **Major Task 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 Task 1

    1) Major activities

    Binding assays were performed using purified HMGB3 protein, demonstrating that HMGB3 binds to 57 base pair DNA duplex and to triplex forming oligonucleotide (TFO) targeted DNA interstrand crosslink (ICL) containing triplex DNA. Further, small molecules identified from a previous virtual screening were used in these binding studies to detect any effect of these molecules in disrupting the HMGB3 and DNA (both duplex and ICL containing triplex DNA substrates) complex formation. We were able to identify a molecule that inhibited HMGB3-DNA complex formation. These data 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.

    2) Specific objectives

    The specific objectives were to determine whether HMGB3 binds to A) double stranded DNA, B) to TFO-directed ICL containing triplex DNA with high affinity, and forms stable complexes *in vitro,* C) to establish this binding assay as a valid experimental approach to identify small molecules that can disrupt HMGB3's high affinity binding to DNA.

    3) Significant results

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

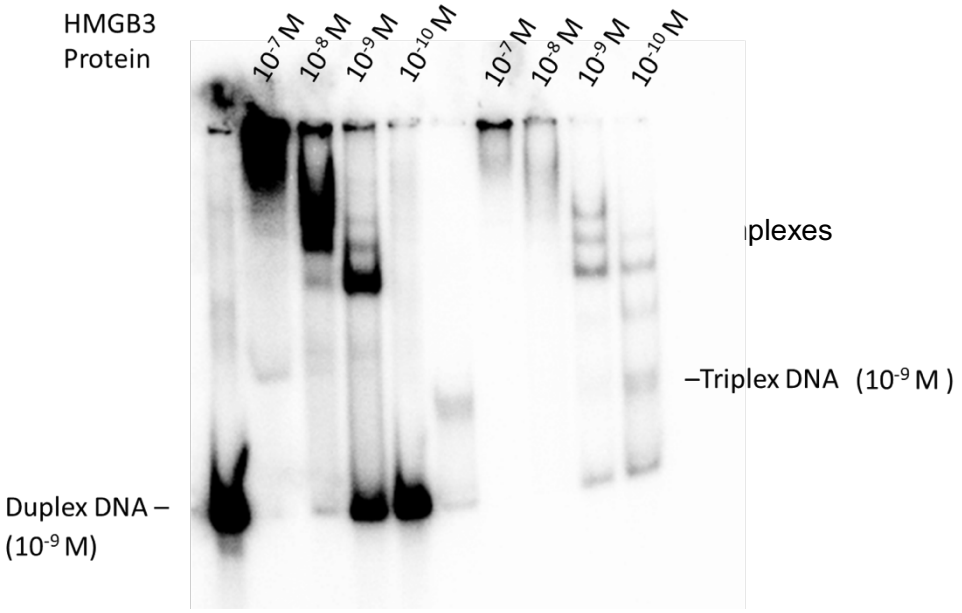

Figure 1: HMGB3 binds to 57 bp duplex DNA and TFO-directed ICL containing triplex DNA.

Goals not met

High through-put screening using 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 complex formation as shown in Figure 1.

Description of the methodology

Fifty-seven base pair duplex DNA was radiolabeled using γ32P ATP. For *in vitro* DNA-protein binding assays, 10^{-9}M duplex radiolabeled 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 triplex DNA, 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/cm^2 UVA to induce DNA interstrand crosslinks. These TFO-ICLs were then used for *in vitro* binding assays to determine HMGB3-triplex complex formation as mentioned above.

Major Task 2

4) Major activities

Institution approved animal protocol was submitted to DoD for ACURO approval. 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 pENTR/THT plasmid vector which contains a H1 promoter that is regulated by tetracycline. Novel interaction between cisplatin resistance-associated overexpressed protein (CROP/LUC7L3) and HMGB3 were verified, role of LUC7L3 in sensitizing cisplatin resistant ovarian cancer cells were studied using siRNA-based methods.

5) Specific objectives

Specific objectives were to generate Tetracycline (or doxycycline) inducible HMGB3 shRNA expressing chemosensitive A2780 and chemoresistant A2780/CP70 ovarian cancer cell lines. These cell lines are going to be used in developing mouse xenografts.

6) Significant results

Chemosensitive A2780 and chemoresistant A2780/CP70 ovarian cancer cells were successfully transfected with TetR expressing pcDNA6/TR plasmid. 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 (**Figure 2**). Four HMGB3 transcript variants were identified. Not all 4 transcript variants were targetable. We found that transcript variants 2 and 4 were only targetable. HMGB3 shRNAs were successfully cloned into tetracycline inducible pENTR/THT vectors.

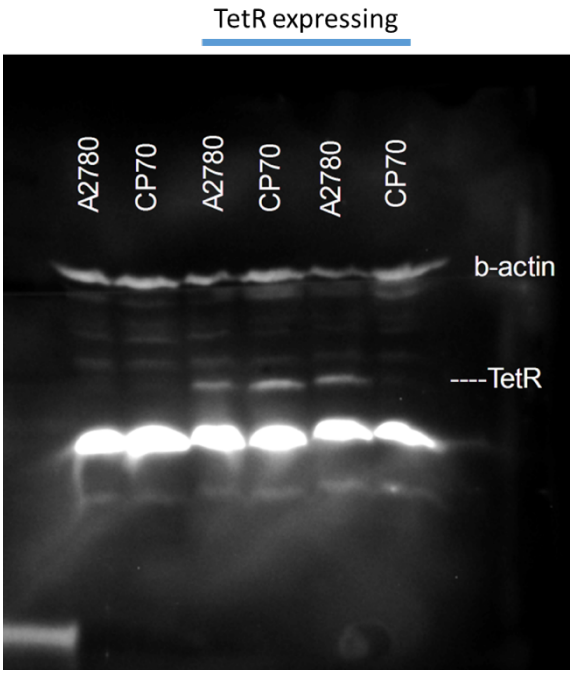

Figure 2: Chemosensitive A2780 and chemoresistant A2780/CP70 cells expressing tetracycline repressor (TetR) protein.

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 spliceosome. We studied the role of targeting LUC7L3 in both A2780 and

A2780/CP70 cells. In the chemosensitive A2780 cells, siRNA mediated depletion of LUC7L3 was lethal, as seen in Figure 3. 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 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.

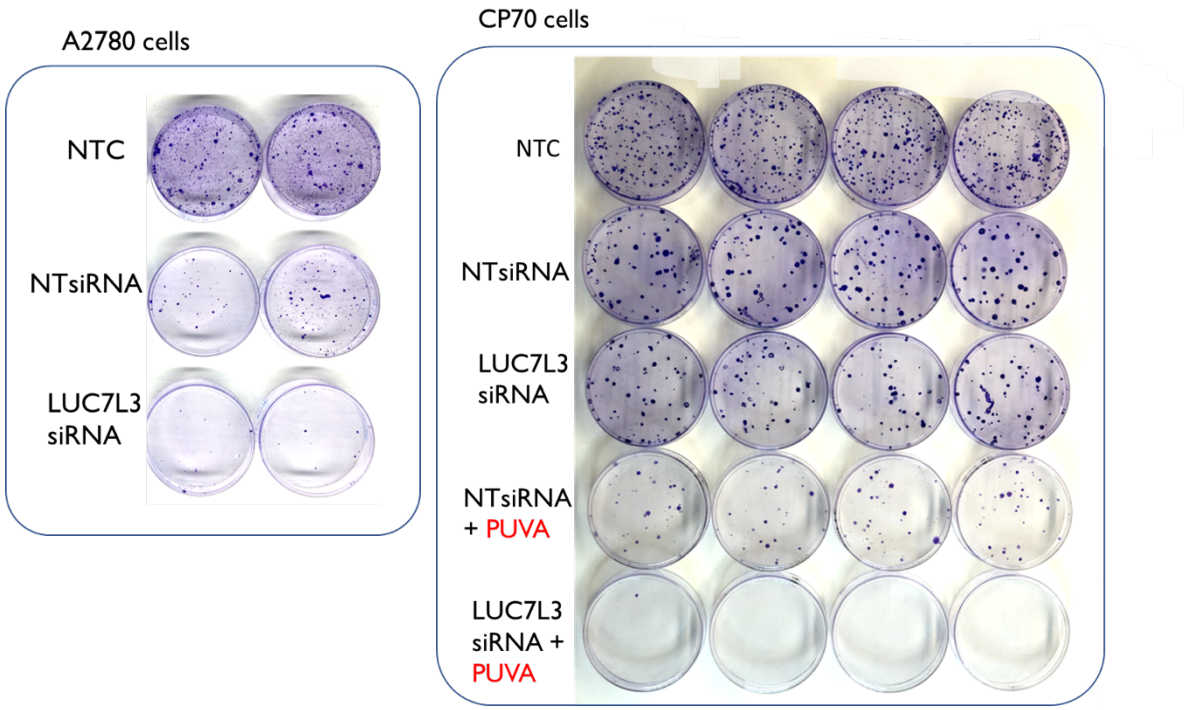

Figure 3: SiRNA-mediated depletion of LUC7L3 sensitizes chemosensitive A2780 and chemoresistant A2780/CP70 cells and may offer an explanation on how HMGB3 modulates the expression levels of DNA damage response kinases.

Goals not met

Chemosensitive A2780 and chemoresistant A2780/CP70 xenografts with inducible hMGB3 shRNA system were supposed to be established in nude mice during this time frame. However, the BLOCK-iT kit that we have originally proposed to use from ThermoFisher got discontinued and we has to design another tetracycline (or doxycycline) inducible vector system. Due to this unforeseen challenge, it took us longer to develop the inducible HMGB3 shRNA constructs.

Description of the methodology

A2780 and A2780/CP70 ovarian cancer cells were grown and maintained in RPMI media with 1% penicillin/streptomycin and 10% bovine serum albumin (BSA) with 5% CO_2 at 37°C. Two µg of tetracycline repressor (TetR) expression vector pcDNA6/TR were 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, plasmid pENTR/THT with a H1 promoter were obtained from Addgene. For directional cloning of the HMGB3 shRNA sequences into the pENTR/THT plasmid, the plasmid was digested using *Bgl*II and *HinD*III 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 manufacturer 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.

.

- **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. Personal interaction with the attendees of the poster session and scientific discussion provided a great opportunity for professional development.

- **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?**

  To accomplish the goals by the next reporting period, the following actions are going to be taken:

1) Ensure that the shRNA constructs are efficiently depleting HMGB3 in A2780 and A2780/CP70 cells 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 interaction.

6) Use 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**
    - In the original proposal, we proposed that the cell lines will be transfected with an inducible HMGB3-shRNA expression vector, using the BLOCK-iT kit available from ThermoFisher (catalog # K4920-00). Transfected cells will then be selected using Zeocin (100 µg/ml) to develop stable cell lines as per the manufacturer's protocol. However, when we wanted to use the BLOCK-iT kit, we found that ThermoFisher has recently discontinued it due to lack of sales. This created a major pause in the proposed workflow. However, we were able to identify doxycycline inducible shRNA expression vectors available from Addgene and completed developing the shRNA vector.
    - With the change of shRNA expressing construct, a new problem can be foreseen. Previously, with the originally proposed BLOCK-iT kit to conditionally express HMGB3 shRNA, the cells were supposed to be selected with blasticidin (to ensure TetR is being expressed) and with Zeocin (to ensure HMGB3 shRNA is being expressed). The current system that we have constructed will require selection with blasticidin only for both TetR and HMGb3 shRNA. This may lead to less than optimum selection.
    - The company that provided modified oligonucleotides, called Midland, went out of business very recently. Midland has been out supplier for over 20 years for the 5'-psoralen attached triplex forming oligonucleotides. We are in the process of identifying other vendors that can provide us with quality modified oligonucleotides that we can use to prepare TFO-directed ICL containing triplexes and continue with the small molecule library screening and binding assays.
- **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**
    - *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

- *Nothing to report*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	*Anirban Mukherjee*
Project Role:	Key Personnel
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Performed HMGB3-DNA *in vitro* binding interactions to determine HMGB3 binds to double and triple stranded DNA, developed TetR expressing two chemosensitive A2780 and two chemoresistant A2780/CP70 cell lines, cloned HMGB3 shRNA transcript variants 2 and 4 in pENTR/THT expression vector, studied the role of HMGB3-interacting cisplatin resistance associated overexpressed protein (CROP/LUC7L3) in sensitizing chemoresistant ovarian cancer cells, preparing report
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

- *"Nothing to Report."*

9. APPENDICES:

ADDITIONAL NOTES:

MARKING OF PROPRIETARY INFORMATION: Data that was developed partially or exclusively at private expense shall be marked as "Proprietary Data" and Distribution Statement B included on the cover page of the report. Federal government approval is required before including Distribution Statement B. The recipient/PI shall coordinate with the COR/GOR to obtain approval. **REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE.** It is the responsibility of the Principal Investigator to advise the COR/GOR when restricted limitation assigned to a document can be downgraded to "Approved for Public Release." **DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS. DO NOT USE WATERMARKS WHEN MARKING DOCUMENTS.**