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TITLE: Determine the Impact of Novel BRCA1 Translation Start Sites on Therapy Resistance in Ovarian Cancer

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

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Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purpose: Recently, PARP inhibitors have emerged as promising agents for the treatment of BRCA1 mutant ovarian cancers. However, similar to platinum, drug resistance is a major clinical hurdle. *Scope:* In this proposal, we aimed to characterize N-terminal deficient BRCA1 proteins contribution to DNA damage repair and their ability to confer resistance to ovarian cancer therapeutics, as well as identify novel small molecules that specifically kill N-terminal deficient BRCA1 protein expressing cells. *Major findings:* We expressed a number of BRCA1 proteins produced from downstream translation start sites that were truncated at the N-terminal region and lacked the RING domain. We show that RING deficient-BRCA1 proteins were hypomorphic, contributing to RAD51 loading, PARPi and cisplatin resistance. The mechanism we describe may not be limited to cancers with BRCA^{1185delAG} mutations and could be relevant to multiple frameshifting 5' located BRCA1 mutations. However, mutations located after Met-297 (c.891) are unlikely to develop resistance through this mechanism, as we show that the next downstream translation start site at Met-531 (c.1593) produced a functionless protein. Additionally, we identified three new small molecules that could be developed as drugs to specifically target PARP inhibitor resistant cancers that express N-terminal deficient BRCA1 proteins.

15. SUBJECT TERMS

BRCA1, ovarian cancer, PARP inhibitors, cisplatin, DNA damage, homologous recombination

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Table of Contents

INTRODUCTION: Hereditary mutations in the *BRCA1* gene predispose carriers to early onset ovarian cancer. The BRCA1 protein is involved in repairing DNA damage induced by DNA damaging chemotherapies such as platinum and PARP inhibitors. BRCA1 mutations often render the protein product dysfunctional, consequently tumors cannot repair DNA damage caused by chemotherapy, and so these types of tumors are highly sensitive to DNA damaging agents. Our current work has uncovered a novel mechanism of resistance to therapy in *BRCA1*-mutant cancer cell lines, warranting further investigation in additional model systems. Our preliminary data suggest that cancer cell lines harboring *BRCA1* mutations utilize new DNA sequences that are downstream of the original mutation to restore BRCA1 protein levels under PARP inhibitor or cisplatin selection pressure. The novel proteins retain more than 90% of the normal BRCA1 product but lack the initial region of protein. We hypothesized that ovarian cancer patients with germline *BRCA1* mutations that are close to the start of the gene restore protein function by utilizing new protein start sites that are downstream of the original deleterious mutation. In turn, restoration of protein function restores DNA repair and provides resistance to DNA damaging chemotherapies. Although the new BRCA1 proteins provide cellular PARP inhibitor and cisplatin resistance, they lack a region of the protein that normally interacts with another important DNA repair protein - BARD1, and do not form BRCA1-BARD1 complexes. We aimed to characterize the role of BRCA1 proteins that do not interact with BARD1 in the DNA damage response. Furthermore, we predicted that under particular conditions of stress or forms of DNA damage, cells may require BRCA1-BARD1 activity for survival; our strategy will uncover small molecules that elicit a cellular dependency on BRCA1-BARD1 activity. Our ultimate objective is to generate strategies for targeting DNA damaging agent chemotherapy-resistant ovarian cancer that can be developed for clinical application.

KEYWORDS:
BRCA1, ovarian cancer, PARP inhibitors, cisplatin, DNA damage, homologous recombination

ACCOMPLISHMENTS:
What were the major goals of the project?

Specific Aim 1: Identify germline *BRCA1* mutations capable of generating drug resistance-inducing N-terminal deficient proteins.	**Timeline**	**Site 1**	**Date of completion**
Major Task 1: Generate cell lines expressing BRCA1 cDNA constructs deficient in AUG1-4.	Months		
Subtask 1: Perform restriction enzyme digests on pENTRA BRCA1-V5 cDNA to remove the first 4 AUG regions.	1-3	Dr. Wang	7.15
Subtask 2: Perform LR Clonase to shuttle BRCA1 cDNA into pLX304 expression construct.	3-4	Dr. Wang	8.15
Subtask 3: Generate lentivirus and infect SNU-251 and UWB1.289 cells with constructs and select for stable cell lines with blasticdin.	4-6	Dr. Wang	10.15
Milestone(s) Achieved: Ovarian cancer cell lines are established that stably overexpress N-terminal deficient BRCA1 proteins.			10.15
Major Task 2: Test the PARP inhibitor and cisplatin sensitivity of N-terminal deficient BRCA1 protein expressing cell lines.			
Subtask 1: Carry out colony formation assays with SNU-251 and UWB1.289 N-terminal deficient BRCA1 protein expressing cell lines with cisplatin and the PARP inhibitor rucaparib.	6-10	Dr. Wang	3.16

Subtask 2: Calculate the concentration of cisplatin or PARP inhibitor required to reduce colony formation by 50% (LC50) in all add back cell lines.	10-12	Dr. Wang/ Dr.Johnson	3.16
Milestone(s) Achieved: Identification of N-terminal deficient BRCA1 proteins that provide PARP inhibitor and/ or cisplatin resistance.			3.16
Specific Aim 2: Characterize the role of N-terminal deficient BRCA1 proteins in the DNA damage response.			
Major Task 3: Characterize N-terminal deficient BRCA1 proteins ability to be recruited to sites of DNA damage.			
Subtask 1: Carry out immunofluorescence studies determining the ability of BRCA1 add back proteins, RAD51 and other proteins that are dependent on BRCA1 for foci formation.	12-16	Dr. Wang	6.16
Subtask 2: Determine the impact of N-terminal deficient proteins on cell cycle checkpoints by flow cytometry and checkpoint proteins by western blot.	16-20	Dr. Wang	10.16
Subtask 3: Identify protein interactions that are specific to N-terminal deficient BRCA1 proteins by mass spectrometry.	12-16	Dr. Wang Dr. Duncan	1.17
Specific Aim 3: Identify small molecules that kill N-terminal deficient BRCA1 protein expressing cells response.			1.17
Major Task 4: Screen FCCC compound library.			
Subtask 1: Optimize screening conditions specific to SNU-251 and UWB1.289 cell lines that express endogenous BRCA1 proteins.	12-16	Dr. Wang	2.17
Subtask 2: Screen FCCC small molecule library against UWB1.289 and SNU-251 cells that express endogenous BRCA1 proteins.	16-20	Dr. Wang	4.17
Subtask 3: Carry out data analysis and identify compounds that specifically kill N-terminal deficient BRCA1 protein expressing cell lines.	20-22	Dr. Wang and Dr. Johnson Dr. Einarson	5.17
Subtask 4: Carry out colony assays on top 10 hits to validate selective killing of N-terminal deficient BRCA1 protein expressing cell lines.	22-24	Dr. Wang/ Dr. Johnson	6.17
Milestone(s) Achieved: Identification and validation of compounds that selectively kill N-terminal deficient BRCA1 protein expressing cell lines.			6.17

What was accomplished under these goals?

We predicted that BRCA1 proteins lacking a short section of the N-terminal region of the protein were capable of providing drug resistance; however, more severely N-terminal truncated proteins are likely to lose essential functional domains and consequently be unable to provide drug resistance. There are many different germline *BRCA1* mutations that are located toward the 5' region of the gene and predispose carriers to early-onset ovarian cancer. In the first Aim of this proposal, we aimed to generate protein products from in-frame translation start sites that are downstream of clinical germline mutations. Proteins were to be assessed for their ability to provide PARP inhibitor and platinum resistance.

Figure 1. BRCA1 cDNA was subject to restriction enzyme digests to remove regions corresponding to translation start sites AUG1-4. Protein products were increasingly deficient of N-terminal amino acids (aa).

Initiation of eukaryotic protein translation requires a minimum nucleotide sequence of AUG. *BRCA1* mRNA consists of 4 in-frame AUG sequences downstream of the assigned start site through to exon 11. We generated all 4 in-frame protein products using a pENTRA BRCA1- C-terminal tagged V5 cDNA construct. Restriction enzyme digests were employed to remove N-terminal regions corresponding to AUG 1-4 of the BRCA1 cDNA, generating increasingly truncated N-terminal deficient BRCA1 proteins (**Figure 1**).

We then shuttled BRCA1 cDNA into pLX304 lentiviral expression construct using the LR clonase Gateway system and lentivirus was generated. In addition to N-terminal deficient BRCA1 constructs, full-length BRCA1 and mCherry cDNA expression constructs were used as positive and negative controls, respectively. SNU-251 and UWB1.289 cells are ovarian cancer cell lines that contain deleterious BRCT domain and exon 11 located *BRCA1* mutations, respectively. We had originally planned on using these cell lines for ectopic add back experiments. However, we found that both cell lines express residual endogenous BRCA1 proteins that have functionality and provide some degree of PARPi and cisplatin resistance. Additionally, these cell lines proved difficult to infect with lentivirus and generate stable cell lines. In place of these cell lines, we have utilized the BRCA1 mutant MDA-MB-436 cell line. This cell line is ideal for measuring the activity of ectopic add back BRCA1 mutant proteins as no endogenous BRCA1 protein can be detected and cells are exquisitely sensitive to PARPi and cisplatin treatments. Furthermore, these cells can be readily manipulated using our lentiviral add back system. In **Figure 2A**, we show MDA-MB-436 cells that were infected with lentivirus containing BRCA1 constructs that initiated translation at Met 1, 48, 128, 297 and 531, as well as mCherry control cells. Additionally, SUM1315MO2 was the original cell line where we detected this phenomenon; we therefore also overexpressed Met 1 and Met297 BRCA1 proteins in this cell line (**Figure 2B**).

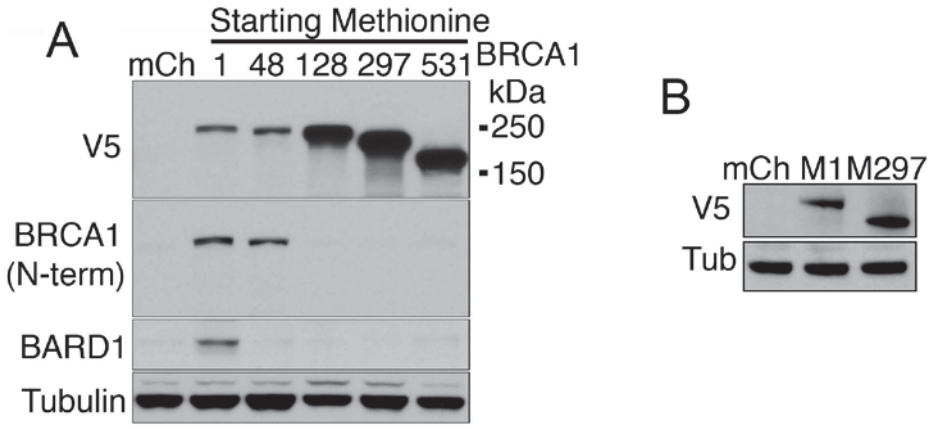

Figure 2. BRCA1 cDNA was subject to restriction enzyme digests to remove translation start sites AUG1-4, so that translation was initiated at Met48, 128, 297, 531. Met1 is full length BRCA1 and mCherry is negative control. (A) MDA-MB-436 ectopic add back cells. (B) SUM1315MO2 ectopic add back cells.

To assess the ability of Rdd-BRCA1 proteins to provide therapy resistance, MDA-MB-436 cells were seeded at a range of densities and incubated with a single rucaparib and cisplatin concentration that selected for drug resistant colonies. Met-1, -48, -128, and -297-BRCA1 expressing cells had 253- ($P = 0.0016$), 36- ($P < 0.001$), 30- ($P < 0.001$), 47-fold ($P = 0.0033$) more rucaparib resistant colonies than mCherry control cells, respectively; as well as 321-, 41-, 55-, 60-fold (all $P < 0.001$) more cisplatin resistant colonies than mCherry control cells, respectively (**Figure 3A**). Interestingly, BRCA1-Met-531 had no impact on PARPi and cisplatin rescue. Furthermore, SUM1315MO2 cells overexpressing exogenous BRCA1 full-length or Met-297 were 101.3- ($P = 0.0255$) and 10.3-fold ($P = 0.0169$) more resistant to rucaparib, respectively; as well as 4.3- ($P = 0.0003$) and 2.1-fold ($P = 0.0004$) more resistant to cisplatin compared to mCherry expressing cells, respectively (**Figure 3B**).

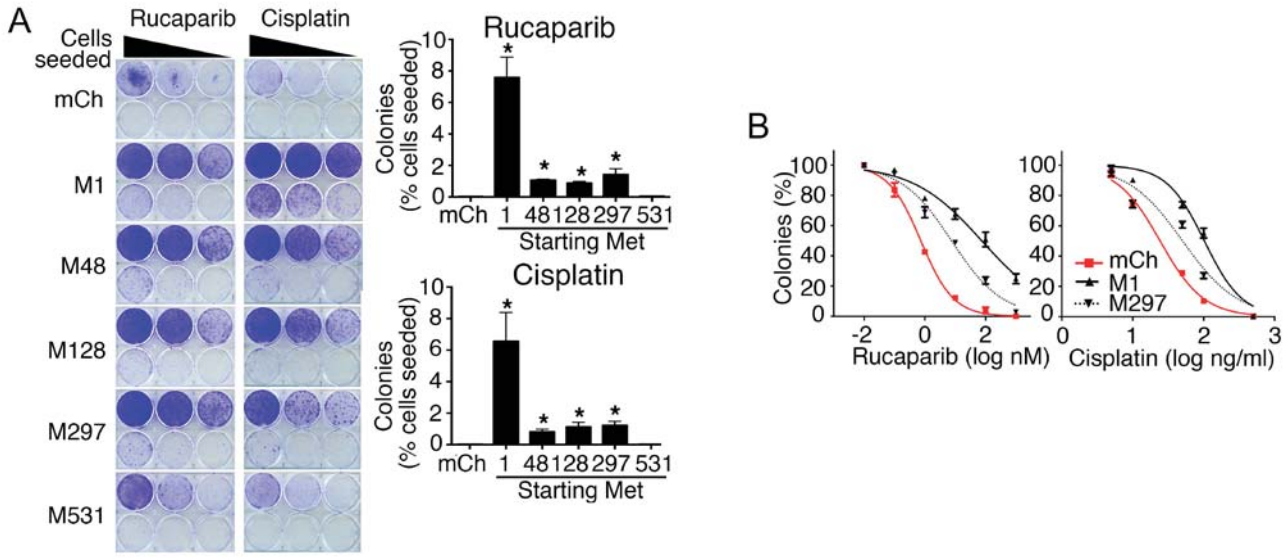

Figure 3. (A) mcherry or M1, M48, M128, M297 or M531 expressing MDA-MB-436 cells were treated with either rucaparib or cisplatin in 6 well plates. Cells were seeded at decreasing densities and the emergence of resistant cells measured. (B) SUM1315MO2 cells expressing mcherry, M1 or M297 BRCA1 were subject to increasing concentrations of either rucaparib or cisplatin and colony formation measured.

In addition to the assays described above, we also carried out standard colony formation assays with the MDA-MB-436 cells. In the graphs below, bars represent LC50 values (concentration required to reduce colony formation by 50% of the control untreated cells). Met-1, -48, -128, and -297-BRCA1 expressing cells had 50.8- (P = 0.0037), 4.8- (P = 0.0066), 4.7- (P = 0.0002), 7.7-fold (P = 0.0034) increased rucaparib LC50 values compared to mCherry control cells, respectively; as well as 2.7- (P = 0.003), 1.6- (P = 0.0034), 1.5- (P = 0.0079), 2.1-fold (P = 0.0008) increased cisplatin LC50 values compared to mCherry control cells, respectively. BRCA1-Met-531 had no impact on PARPi and cisplatin rescue (**Figure 4**).

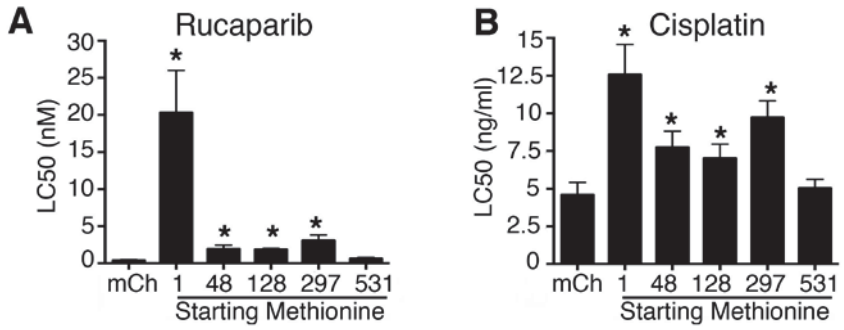

Figure 4. MDA-MB-436 cells expressing mcherry or M1, M48, M128, M297 or M531 BRCA1 were subject to increasing concentrations of either rucaparib or cisplatin and colony formation measured. Bar graph shows LC50 concentrations (concentration required to reduce colony formation by 50%).

We also determined the impact of RING deficient BRCA1 proteins on the DNA damage response kinetics and measured BRCA1, RAD51 and H2AX irradiation induced foci (IRIF). BRCA1-Met-1, -48, -128, -297, but not Met-531, formed efficient BRCA1 IRIF. However, BRCA1-Met-48, -128, -297 and -531 had 1.4- (P = 0.026), 1.6- (P = 0.0138), 1.5- (P = 0.0671) and 7.3-fold (P = 0.0023) less RAD51 IRIF compared to BRCA1-Met-1 (full-length) expressing cells. The levels of γ-H2AX as were not affected by exogenous protein expression (**Figure 5**).

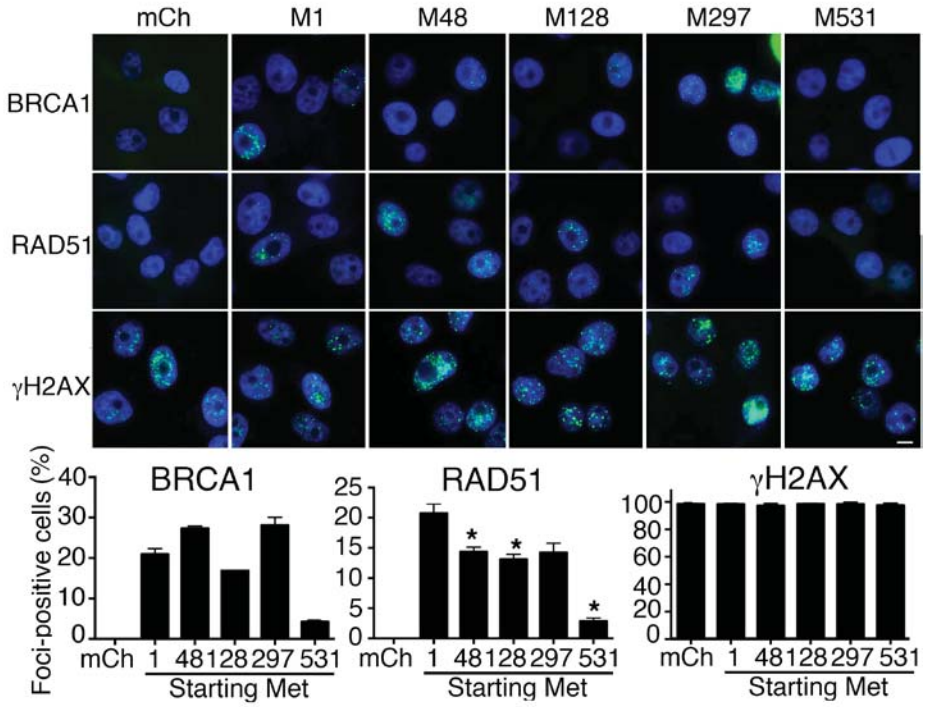

Figure 5. MDA-MB-436 cells described above were subject to irradiation and BRCA1, RAD51 and H2AX foci formation measured by immunofluorescence. Representative pictures are shown and mean number of foci positive cells.

We have further characterized the DNA repair capacity of RING domain deficient BRCA1 proteins in MDA-MB-436 cells. We were interested in the ability of RING domain truncations to contribute toward the DNA damage checkpoint response. BRCA1 has previously been shown to be part of G2/M DNA damage checkpoint cell cycle arrest. We investigated G2/M checkpoint arrest by flow cytometry in MDA-MB-436 cells that expressed mCherry control, wild-type BRCA1, Met297 or Met531 N-terminal truncations. As expected, BRCA1 null cells that express mCherry did not demonstrate G2/M cell cycle arrest 24 hours after 10 Gy IR exposure. However, BRCA1 WT add back cells had a robust G2/M cell cycle arrest. Furthermore, Met297 BRCA1 add back cells also demonstrated a strong G2/M arrest, but Met531 expressing cells failed to demonstrate any cell cycle arrest (**Figure 6**). Therefore, we believe that the peptide region spanning Met297 to Met531 is critical for checkpoint arrest but RING domain deficient proteins up to Met297 can contribute to the DNA damage checkpoint to a similar degree as full-length BRCA1 proteins. Of note, we carried out mass spectrometry after immunoprecipitating Met-297 from MDA-MB-436 cells and compared protein-protein interactions to full-length BRCA1 expressing cells. As expected, full length BRCA1 samples had readily detectable BARD1 peptides, however Met297 had undetectable BARD1 peptides. No other significant differences between the samples could be detected, suggesting that besides the RING domain mediated BARD1 interaction, Met-297 has similar protein-protein interactions as full-length BRCA1.

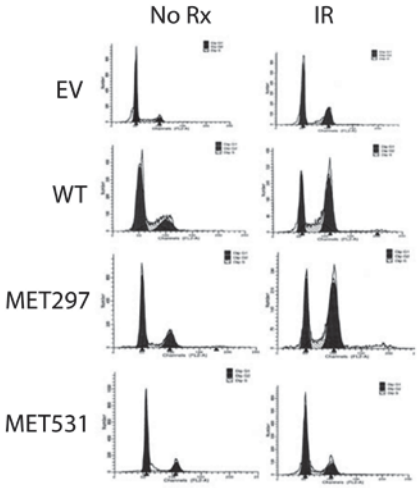

Figure 6. MDA-MB-436 cells that express either mCherry, WT, Met297 or Met531 BRCA1 were treated with 10 Gy IR or no treatment (no rx) and 24 hours later collected for cell cycle analyses by flow cytometry.

UWB1.289 and SNU251 ovarian cancer cell lines that are BRCA1 mutated were engineered to express full-length or Met297 BRCA1. Cells were used to screen the FCCC small molecule compound library with the aim of identifying compounds that specifically kill Met297 but not full-length BRCA1 expressing cells. Three separate 96-well plates of compounds were assessed for their effect on the viability of cell lines over a 5-day incubation period. Cell titer-glo was used to measure viability. The majority of compounds either had no impact or reduced the viability of full-length and Met297 BRCA1 add back cells to a similar degree. However, we were able to identify several compounds that reduced the viability of Met297 expressing cells by more than 30% of the reduction observed in full-length BRCA1 add back cells (**Figure 7**). These compounds were selected for further analyses.

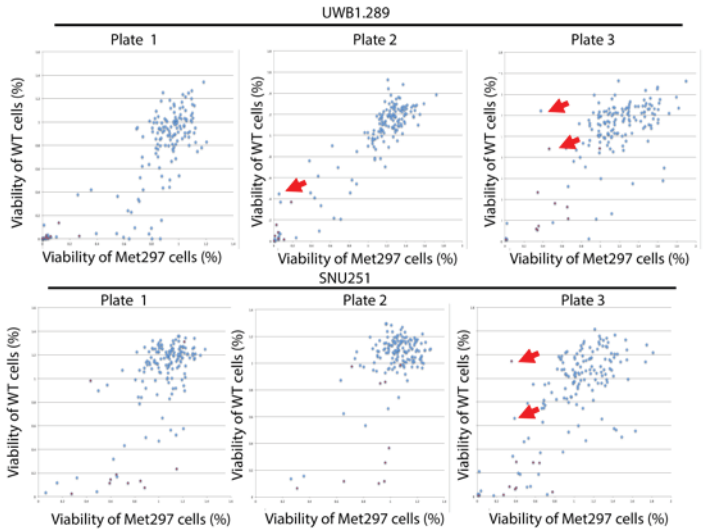

Figure 7. UWB1.289 and SNU-251 cells that express WT (y-axis) or Met297 (x-axis) were treated compounds from the Fox Chase Cancer Center small molecule compound library and assessed for cell viability after 5 day incubation. Compounds that reduced the viability of Met297 cells more than 30% of WT add back cells were selected for further analyses and are indicated with red arrows.

We next carried out colony formation assays using the top 6 hit compounds where cells were subject to increasing concentrations of compound and assessed for ability to form colonies over a 2 week incubation period. Compounds 1-6 were previously identified as inhibitors of Cdks as well as cell signaling proteins (**Table 1**). We found that compounds 1, 4 and 6 validated as being potent inhibitors of Met297 but not full-length BRCA1 expressing cell lines (**Figure 8**). In future studies, beyond the scope of this grant. We will carry out xenograft assays as well as investigate the biological mechanism by which these compounds kill Met297 expressing cells.

Hit#	Target
1	Cdk4/D1
2	Flt
3	p60, PDGF, c-Src
4	MK2a
5	Src, Cdk1/E, Cdk2/A, Cdk1/B
6	NFkB, TNFalpha

Table 1. Hit compounds and the previously identified protein targets.

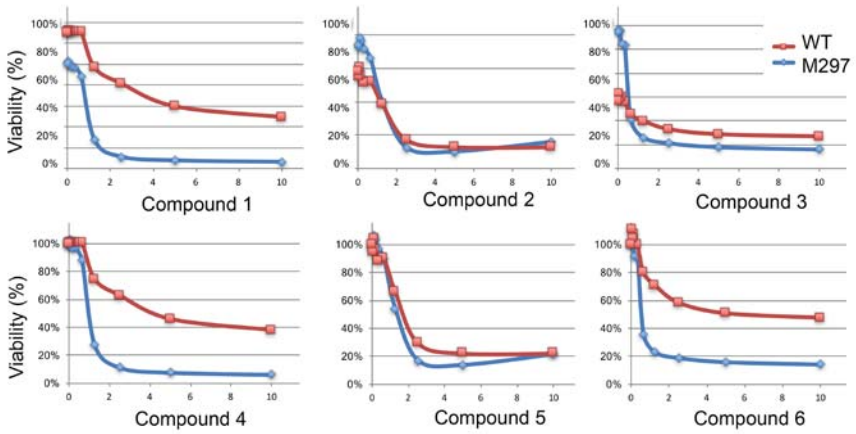

Figure 8. UWB1.289 cells that express WT (red) or Met297 (blue) were treated with hit compounds identified above and assessed for their impact on colony formation after a 2 week incubation.

What opportunities for training and professional development has the project provided?
Dr. Yifan Wang is a postdoctoral researcher and the Teal Scholar associated with this project. During the grant period Dr. Wang has attended and presented data at Fox Chase Cancer Center gynecologic cancer meetings. Dr. Wang attended and presented his work at the international AACR meeting 'DNA Repair: Tumor development and therapeutic response' in Montreal Canada, November 2016. Since the award, Dr. Wang has increased his interactions with ovarian cancer researchers and physicians at the center, including Drs. Denise Connolly, Lainie Martin and Stephen Rubin. Additionally, Dr. Wang has attended Faculty, Molecular Therapeutics and biweekly Invited Speaker Seminars. Dr. Wang has also presented data at Johnson lab meetings as well as at the FCCC Post-doctoral Seminar series, FCCC Postdoc Day, and Temple Biomedical Research Day. FCCC's recent affiliation with Temple University has enabled Dr. Wang to attend courses and classes. Dr. Wang attended Medical Pharmacology (500) and Biostatistics (5312) classes. Dr. Wang also regularly met with his mentor Dr. Johnson for one to one discussions and guidance. Dr. Wang has also been working on preparing additional manuscripts that will be published in the next 12 months and will acknowledge the support of this grant. We anticipate Dr. Wang will begin to apply for faculty positions and will set up his own independent laboratory where he will focus on ovarian cancer therapeutics in the next 12-24 months.

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? – Nothing to Report.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Tumor resistance to platinum therapy is the primary cause ovarian cancer related death. This proposal is currently underway and in the process of revealing biological mechanisms that transform drug sensitive tumors to those that no longer respond to therapy. Understanding why tumors become drug resistant is paramount for the design of new strategies that effectively target drug resistant tumors and reduce ovarian cancer deaths. Ovarian tumors usually respond well to first-line platinum based therapies, only for resistance to develop, followed by disease progression and eventual cancer-related death. The mechanisms of platinum resistance are not well characterized, despite several decades of research. Our work shows that resistance mechanisms are diverse, dependent on the original platinum-sensitizing genetic lesion, and in the case of *BRCA1*, allele-specific drug resistance mechanisms exist. To improve the current understanding of ovarian cancer drug resistance so that resistant tumors can be effectively treated, mutation-focused studies are crucial and will provide a foundation for the rational design of approaches that target resistant disease. PARP inhibitor therapy is an exciting new treatment option for ovarian cancer patients. Our work has shed light on understanding the clinical limitations and long-term effects on ovarian cancer cells and tumors. The observation that *BRCA1* mutant tumors harboring N-terminally located frameshift mutations will likely develop identical mechanisms of resistance to PARP inhibitors and platinum based therapies, suggests drug cross-resistance should be taken into account. These results have important, immediate implications for the clinical management and application of PARP inhibitor and platinum therapies. When either platinum or PARP inhibitor resistance develops, patients with N-terminal specific BRCA1 allelic mutations may benefit from alternative therapies with different mechanisms of action. Furthermore, our work enables the mechanism of drug resistance to be predicted, based on the location of patients' *BRCA1* mutation. Additionally, we have now identified new small molecules that could be developed as drugs to specifically target PARP inhibitor resistant cancers that express N-terminal deficient BRCA1 proteins.

What was the impact on other disciplines? – Nothing to Report.

What was the impact on technology transfer? – Nothing to Report.

What was the impact on society beyond science and technology? – Nothing to Report.

CHANGES/PROBLEMS: Nothing to Report.

PRODUCTS:

Publications, conference papers, and presentations

Journal publications.
Wang Y, Krais JJ, Bernhardy AJ, Nicolas E, Cai KQ, Harrell MI, Kim HH, George E, Swisher EM, Simpkins F, Johnson N. BRCA1 RING Domain-Deficient Proteins Promote PARP Inhibitor and Platinum Resistance. *J Clin Invest*. 2016 Aug 1;126(8):3145-57. PubMed PMID: 27454289 Acknowledgement *of federal support (yes).*

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.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	***Neil Johnson, Ph.D.***
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Management and oversite*
Funding Support:	*Not Applicable*
Name:	***Yifan Wang, Ph.D.***
Project Role:	Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Generation of construct, cell culture, experiments proposed*
Funding Support:	*Not Applicable*
Name:	***Andrea Bernhardy***
Project Role:	Scientific Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*8*
Contribution to Project:	*Preparation of reagents, cell culture, experiments proposed*
Funding Support:	*Not Applicable*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? – Not Applicable.

What other organizations were involved as partners? – Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

Collaborative Awards – Not Applicable.

Quad Charts – Not Applicable.

APPENDICES – Not Applicable.