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PRINCIPAL INVESTIGATOR: Dr. Shunqiang Li, PhD

CONTRACTING ORGANIZATION: Washington University

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Dr. Shunqiang Li, PhD

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The Washington University
Campus Box 8076
660 South Euclid Avenue
St. Louis, MO 63110

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14. ABSTRACT

Estrogen receptor alpha (ERα) is expressed in ~70% of all human breast cancers and, therefore, is a major therapeutic target for endocrine therapy. The lack of response to anti-estrogens is a hallmark of resistance to endocrine therapies, yet the mechanisms are not completely understood. One emerging mechanism is the development of mutations in *ESR1*, the gene encoding ERα. These mutant ERα proteins confer significantly higher ERα activity than the wild-type receptor and are resistant to degradation by selective estrogen receptor degraders (SERDs) such as faslodex. Our laboratory discovered a natural plant product, Diptoindonesin G (Dip G), that significantly decreases ERα levels. We determined that Dip G functions via Hsp90α /CHIP, yet the mechanism is different from that of the Hsp90α ATPase inhibitor 17-AAG. Dip G is more effective than faslodex in inhibiting growth of ERα mutant cell lines, with the levels of degradation of ERα, inhibition of ERα target genes and inhibition of cell proliferation all being concordant. Moreover, we have shown that Dip G can effectively inhibit the growth of human tumors in mice models without causing detectable tissue damages. Thus, Dip G may have minimum adverse side effects on normal tissues at the therapeutically effective doses. This application will test the hypothesis that Dip G alone or Dip G in combination with SERDs are effective to treat hormone-resistant breast cancers, including those harboring the *ESR1* mutations.

15. SUBJECT TERMS

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1. INTRODUCTION:

Our goal is to develop Diptoindonesin G (Dip G) into an effective therapeutic drug for treating mutant ERα expressing, endocrine-resistant breast cancers. We will elucidate the mechanism of action of Dip G and evaluate the anti-cancer effects of Dip G in endocrine-resistant cell lines, organoids, and patient derived xenograft (PDX) models harboring *ESR1* mutations, in comparison with other clinically-investigated ER degrading agents.

2. KEYWORDS:

Endocrine resistance, Estrogen Receptor, Diptoindonesin G, Selective Estrogen Receptor Degrader (SERD), Breast Cancer, Patient-Derived Xenograft

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Goal 1: Mechanistic study of Dip G action and the dependency of cytotoxic effects on CHIP, Hsp90, and ER protein levels. We have established CHIP KO MCF7 and MCF7-ERY537S cell lines and performed proteomics studies to determine CHIP-dependent global protein changes. Fluorescence polarization assay was established to measure the binding affinity of Dip G with recombinant Hsp90, CHIP and ER (30% completion).

Goal 2: Assess the effects of Dip G and SERDs in tumor organoids and MCF7 xenograft mouse models. We have established organoids for WHIM9, 11, 18 and 20, which represent both wildtype ER and mutant ER expressing tumors. We have generated MCF7-luciferase reporter cell line for xenograft experiments (20% completion).

Goal 3: Test the anti-cancer effects of Dip G, Deoxy-Dip G, Faslodex and their combination in PDX tumor models. We have characterized the metastatic features of WHIM 9, 11, 18, and 20 by tail-vein injection (20% completion).

What was accomplished under these goals?

(1) Major activities:

Specific Aim 1: Mechanistic study of Dip G action and the dependency of cytotoxic effects on CHIP, Hsp90 and ER protein level.

Major Task 1: Determine correlation of Dip G cytotoxicity with CHIP, Hsp70 and ER protein levels. We have successfully generated CHIP KO MCF7 cell line and measured the dependency of CHIP for Dip G and 17-AAG-induced ER degradation. Interestingly, CHIP protein is not required for either Dip G or 17-AAG induced ER degradation.

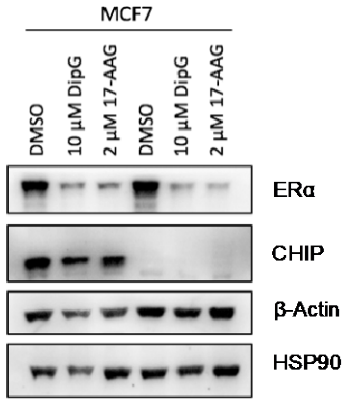

Figure 1. Dip G and 17-AAG induced ER degradation is independent of CHIP expression. Western blotting of ER after treatment of parental and CHIP KO MCF7 cells with Dip G (10 μM) or 17-AAG (2 μM) for 24 hours.

Major Task 2: Determine the binding of Dip G to Hsp90/ER/CHIP complex

We have developed fluorescence polarization assay to measure the binding affinity of Dip G to Hsp90, CHIP and ER. Interestingly, Dip G and 17-AAG (geldanamycin) binding affinity to recombinant Hsp90 were measured to be 350 nM and 509 nM, respectively, suggesting that Dip G directly binds to Hsp90. On the contrary, Dip G has weak binding to CHIP and ER recombinant proteins. This result suggests that Dip G likely modulates ER protein degradation through binding to Hsp90 as a molecular glue to CHIP and ER. 17-AAG is known to bind to the ATPase domain of Hsp90. It is unknown which domain(s) of Hsp90 binds to Dip G. For this reason, we did not pursue the cytotoxicity experiment with ER overexpression. Rather, we focus on Major task 3 to compare the global protein changes by Dip G and 17-AAG.

Protein	Drug/Peptide	K_D (Dissociation Constant)
HSP90	Geldanamycin-HSP90 inhibitor	509 nM
HSP90	Deoxy-DipG	349 nM

Figure 2. Binding affinity of Dip G to Hsp90 measured by fluorescence polarization.

Major Task 3: Identify CHIP-dependent global protein changes by Dip G in MCF7 cells.

We have performed the proteomics analyses comparing Dip G with Hsp90 inhibitor 17-AAG. Our results showed that Dip G regulated proteins significantly overlap and constitute a subset of proteins regulated by 17-AAG. These results further substantiate the mechanism of action of Dip G is linked to regulation of Hsp90 activity.

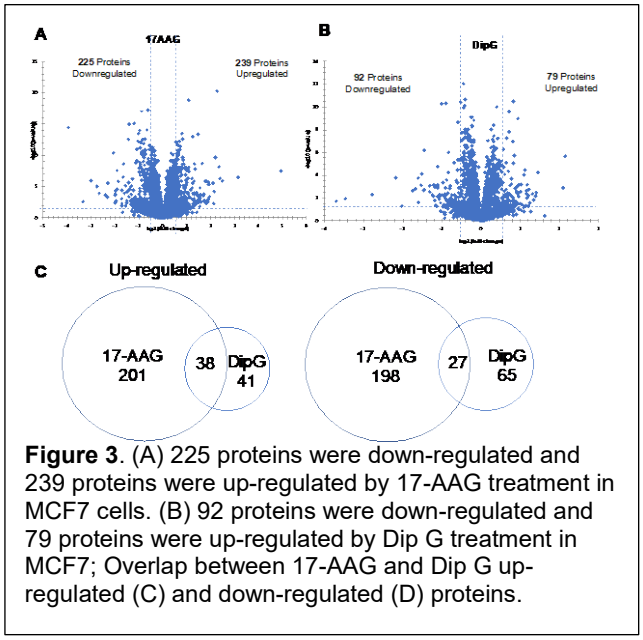

Figure 3. (A) 225 proteins were down-regulated and 239 proteins were up-regulated by 17-AAG treatment in MCF7 cells. (B) 92 proteins were down-regulated and 79 proteins were up-regulated by Dip G treatment in MCF7; Overlap between 17-AAG and Dip G up-regulated (C) and down-regulated (D) proteins.

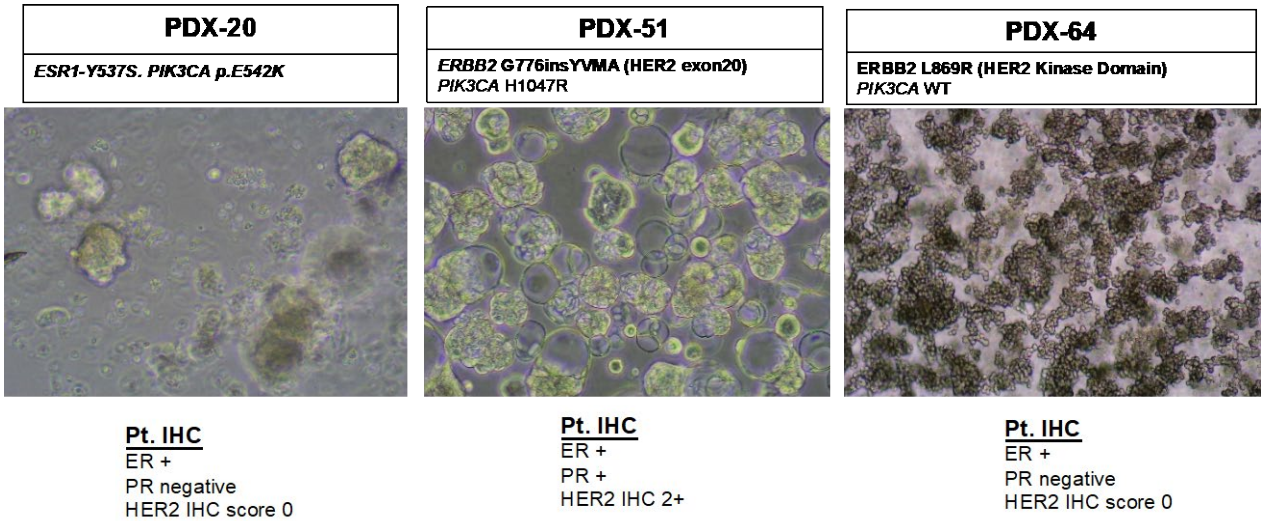

Figure 4. Establishment of ER+ PDXs for determining the cytotoxic effects of Dip G. The morphology of organoids for WHIM-20, 51 and 64 with differential expression of ER, PR and HER2 are shown.

Specific Aim 2: Assess the effects of Dip G and SERDs in tumor organoids and MCF7 xenograft mouse models.

Major Task 4:
Dr. Li has established multiple organoids from ER+ PDXs (Figure 4). WHIM20/PDX20 expresses ERY537S. These organoids will be used for examining the effects of Dip G and SERDs.

Major Task 6:
In order to better quantify the effects of drugs on the growth of MCF7 xenograft tumors, we stably expressed GFP-luciferase in MCF7 ERY537S cells. 1x 106 cells in PBS and Matrigel were injected into the mammary fat pads on either side of each mouse. Tumor size was measured weekly and mice were imaged using IVIS weekly by i.p. injection of luciferin substrate into each mouse (100 ul/mouse) 10 minutes prior to imaging. Both the primary tumors and lungs were imaged. When tumors reached the threshold size for treatment, mice (n=10) were randomized to treatment groups such that the average size for each group was approximately 100mm^3. Dip G (40mg/kg in PEG400 & 0.9% saline) was administered daily by s.c. Vehicle (DMSO + PEG400 & 0.9% saline) treatment group was administered daily by s.c., Fulvestrant (150mg/kg in corn oil) was administered weekly by s.c. The treatment was lasted for 12 weeks before sacrifice. Unfortunately, we did not observe changes in tumor size and luciferase signals in response to treatment regimens among groups (Figure 5). The reason for this remains unclear, because even Fulvestrant treatment did not inhibit tumor growth.

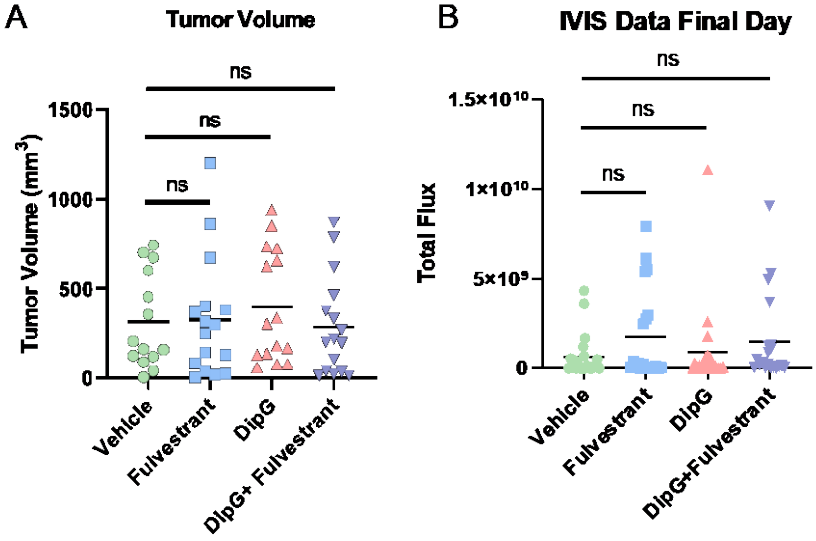

Figure 5. (A) No difference was observed in tumor volume means among treatment groups after 12 weeks. (B) No difference was observed in luciferase signals among treatment groups.

Specific Aim 3: Test Dip G and SERD's effects in PDX tumor models.

Major task 8: Measure in vivo anti-cancer effects of Dip G and SERDs in PDX models
Subaim 1: establish PDX model.
Dr. Li has been working on the metastatic features of WHIM 9, 11, 18, and 20 by tail-vein injection. PDX cells developed metastasis in multiple sites in mice (Figure 6).

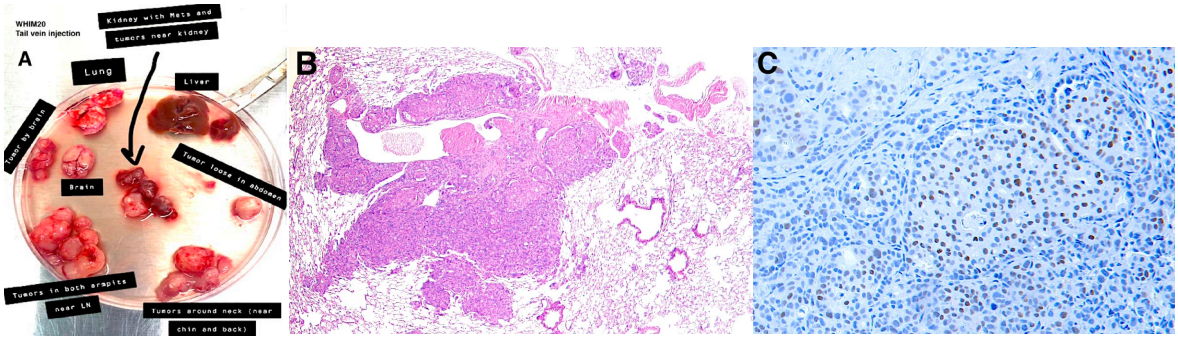

Figure 6. Development of metastasis after tail vein injection of WHIM20 in NSG mice. (A) Multiple gross metastasis. (B) Lung, H&E staining. (C) Lung, IHC for ER.

Specific objectives: Elucidate the mechanism of action of Dip G and investigate the therapeutic effects of Dip G in organoids and PDX models *in vivo*.

(2) Significant results and major findings: (a) Towards understanding the mechanism of action of Dip G, we performed fluorescence polarization assays to measure the binding affinity of Dip G to recombinant proteins Hsp90, CHIP and ER. The results showed that Dip G has the strongest binding affinity to Hsp90 (Figure 2), probably serving as a molecular glue for CHIP and ER. We then performed proteomics analyses comparing Dip G and Hsp90 inhibitor 17-AAG. The results showed that Dip G affected proteins represent a subset of 17-AAG regulated proteome (Figure 3), suggesting that Dip G functions through Hsp90/CHIP but is distinct from 17-AAG. (b) Using CHIP KO MCF7 cells, we found that Dip G-induced ER degradation still persists in CHIP KO MCF7 cells, suggesting that other ubiquitin ligase E3 may complement CHIP function when CHIP is depleted. To identify Dip G binding partner proteins, Dr. Tang has synthesized Dip G alkyne derivatives for click-chemistry and pull-down direct Dip G interacting partner proteins using streptavidin beads. Our preliminary experiments identified CHIP and Hsp90 in Dip G analogue pull-down experiments. We will repeat the pull-down experiments using CHIP WT and KO cell lines, digest the immunoprecipitants with trypsin, and subject the samples for proteomics analyses. (c) We have attempted to establish MCF7 xenograft model for Dip G treatment. Unfortunately, we did not observe any growth inhibitory effect by s.c. administration of Dip G using this model. The negative result could be due to insufficient serum and intratumoral levels of Dip G by s.c. administration. The positive control fulvestrant also did not show growth inhibitory effect. The reason for not observing growth effects by fulvestrant was unknown. Further optimization of Dip G administration is needed.

(3) Other achievements: None

The research activities were impeded by the pandemic. The research labs were shut down between March and June. The research capacity was reduced in the subsequent months to maintain social distancing.

What opportunities for training and professional development has the project provided?
Donahue K, Xu, W. "Diptoindonesin G, a novel ER alpha degrader for the treatment of endocrine resistant cancer" Oral Presentation, and Poster Presentation. Hormone Dependent Cancers Gordon Research Conference, August 5th, 2019

Kristine Donahue, a graduate student in the Xu lab, has participated in on-campus poster sessions with the Science and Medicine Graduate Research Fellows (SciMed GRS) community.

Kristine Donahue was selected into Heidi Dvinge and Patti Keely Trainee Honor Society in 2020

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) Determine the mechanism of action of Dip G. We will identify Dip G protein partner(s) and verify the requirement of Dip G-interacting proteins in ER degradation.
2) Determine the effect of Dip G and its combination with other FDA-approved drugs in ER+ organoids. We will characterize the biological effects of Dip G in organoid models and transcriptional effects of Dip G in different cell types using single-cell transcriptome analyses. This experiment will instruct us to select appropriate PDX for in vivo experiment.
3) Determine the in vivo effects of Dip G in wild-type and mutant ER expressing WHIM models

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
While endocrine therapy has considerably reduced mortality from breast cancer, resistance to this treatment remains a major clinical challenge. Positive outcomes from these studies will lead to the development of Dip G and its analogs as new therapeutic agents for overcoming endocrine-resistance in breast cancers.

What was the impact on other disciplines?
Our proteomics analyses showed that Dip G regulates a subset of Hsp90 inhibitor 17-AAG regulated proteins. Dip G does not appear to have strong cytotoxicity to normal cells as pan-inhibitors of Hsp90. The results suggest that Dip G may substitute Hsp90 inhibitors for treatment of multiple human cancer types including those that have developed treatment resistance.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
In addition to the mammary duct protocol, Dr. Shunqiang Li's group has tried tail vein injection of WHIM 20 which has the advantage of accurately injecting a given number of PDX cells into mice.

The preliminary results showed that there was metastasis in the lung, lymphatic system, and the kidney in mice that received tail vein injection of WHIM20 (Figure 6). The metastasis to organs/tissues were analyzed by IHC staining.

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

We completed most of the tasks in SOW. There was a delay in proteomics in Major task 3. We plan to compare Dip G-affected proteome in parental MCF7 and MCF7 CHIP KO cells. This was delayed due to close of proteomics facility during pandemic.

Administration of Dip G to mice to obtain therapeutically effective dose of the drug remains to be a challenge. We have compared three routes of administration: i.p., subcutaneous, and oral gavage. The subcutaneous and i.p. gave low uM concentrations of Dip G after 1 hour of administration. This is at the low end of the therapeutically effective dose of Dip G in vitro. To overcome this problem, Dr. Tang's group has synthesized Deoxy-DipG, an analogue that is more effective to degrade ER than parental compound. We will test this analogue in the *in vivo* experiments in the future.

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
None

Significant changes in use or care of vertebrate animals
None

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.
Kristine Donahue, Wei Xu "Therapeutic Strategies to Target Activating Estrogen Receptor alpha Mutations", Submitted, *Nuclear Receptors*, SpringerNature Publisher

Other publications, conference papers and presentations.
Donahue K, Xu, W. "Diptoindonesin G, a novel ER alpha degrader for the treatment of endocrine resistant cancer" Oral Presentation, and Poster Presentation. Hormone Dependent Cancers Gordon Research Conference, August 5th, 2019

- **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**

CHIP KO MCF7 cell lines were generated using CRISPR/Cas9

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

WEI XU LAB, UNIVERSITY OF WISCONSIN-MADISON

Name: Wei Xu
Project Role: PI
Researcher Identifier: 0000-0003-3808-0045
Nearest person month worked: 2
Contribution to Project: The PI is responsible for the overall administration and scientific direction of the project.
Funding Support: N/A

Name: Yidan Wang
Project Role: Research Specialist
Researcher Identifier: N/A
Nearest person month worked: 7
Contribution to Project: Ms. Wang assists with growing various tissue culture cell lines for in vitro experiments (Aim 1), generates, selects and maintains the stably transfected cell lines (Aim 2), and performs cell line xenograft animal experiments to study if Dip G treatment inhibits tumor growth (Aim 3) in comparison with SERDs. She is also responsible for ordering supplies and other general lab management duties.
Funding Support: N/A

Name: Dr. Ang Gao
Project Role: Research Associate
Researcher Identifier: N/A
Nearest person month worked: 12
Contribution to Project: Dr. Gao has been working on establishing organoid models using PDX. She will perform high throughput drug screening in organoids (Aim 2).
Funding Support: N/A

Name: Ngai Ting Chan (Steve)
Project Role: Research Assistant
Researcher Identifier: N/A
Nearest person month worked: 9
Contribution to Project: Steve has assisted with cell culture and the mass spectrometry experiments.
Funding Support: N/A

Name: Shengjie Zhang
Project Role: Research Scientist
Researcher Identifier: N/A
Nearest person month worked: 8
Contribution to Project: Dr. Zhang has been working on characterizing the Dip G analogue in cell-based assays.
Funding Support: N/A

Name: Dr. Haibo Xie
Project Role: Assistant Scientist
Researcher Identifier: 2-1350-9557
Nearest person month worked: 10
Contribution to Project: Dr. Xie is responsible for the necessary chemical synthesis in all aims and the mechanism of action studies in aim 1a in the proposal.
Funding Support: N/A

Name: Kristine Donahue
Project Role: Graduate Student
Researcher Identifier: N/A
Nearest person month worked: 2
Contribution to Project: Kristine is the main driver of Dip G project. She performs cell proliferation, ER degradation and RNA-seq experiments. She has also been working on PDX xenograft models and testing the activity of Dip G in vivo.
Funding Support: N/A

SHUNQIANG LI LAB, WASHINGTON UNIVERSITY IN ST. LOUIS, MISSOURI

Name: Shunqiang Li – no change

Name: Julie Belmar, no change

Name: Rose Tipton, new person
Project Role: Research Technician II
Researcher Identifier: 373679
Nearest person month worked: 6 months
Contribution to Project: maintain the PDX cells and re- engraft the PDX cells into NOD mice.
Funding Support: NIH U54 CA224083

Name: Tina Primeau, left
Project Role: Lab manager
Researcher Identifier: 091623
Nearest person month worked: 9 months
Contribution to Project: supervise and work together with the Research Technician II to expand the existing breast cancer PDXs.
Funding Support: NIH U54 CA224083

Name: Amanda Mahoney, left
Project Role: Research Technician II
Researcher Identifier: 391407
Nearest person month worked: 5 months
Contribution to Project: conduct injections to generate tumor-bearing mice
Funding Support: NIH U54 CA224083

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: Shunqiang Li, Partnering PI, will submit a duplicative report.

QUAD CHARTS: N/A

9. APPENDICES: N/A