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PRINCIPAL INVESTIGATOR: Ruiwu Liu, Ph.D.

CONTRACTING ORGANIZATION: The Regents of the University of California Davis

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6. AUTHOR(S)
Ruiwu Liu, Ph.D.; Paramita Ghosh, Ph.D.; Malvina Tsamouri, D.V.M., Ph.D.
Tsung-Chieh Shih, Ph.D., Rebecca Britt Armenta
Huanyao Gao, Ph.D.; Liewei Wang, MD, Ph.D.

E-Mail: rwliu@ucdavis.edu

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Regents of the University of California Davis
1850 RESEARCH PARK DR,
STE 300
DAVIS CA 95618-6153

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14. ABSTRACT
In Year 3 of this award, we investigated whether S-LLS133 is more effective in expressing nuclear or cytosolic, and whether treatment of S-LLS133 induces nuclear localization of Gal-1. Both C4-2B cells that express low levels of Gal-1 and 22Rv1 cells that express high levels of Gal-1 demonstrated enhanced expression of Gal-1 upon treatment with S-LLS133. Moreover, Gal-1 localized to the nucleus upon treatment with S-LLS133 for 72 hours at 1 μM. To evaluate the effects of S-LLS133 on the response to cabazitaxel, we tested both cabazitaxel and S-LLS133 in organoids derived from abiraterone-resistant prostate cancer PDX tumors expressing different levels of galectin 1. S-LLS133 sensitized cabazitaxel-resistant tumors to this chemotherapeutic agent. We performed PK study on S-LLS133 and discovered that the compound is rather stable *in vivo*. We performed a Maximum Tolerated Dose (MTD) experiment to identify the highest dose of S-LLS133 that can be safely administered to mice**.** No signs of toxicity were observed in mice that received S-LLS133 at 10-50mg/kg for 5 consecutive days. Taken together, this suggests that S-LLS133 may be a good drug to target gal-1 sensitive castration resistant prostate cancer.

15. SUBJECT TERMS
Galectin-1, castration resistant prostate cancer, galectin-1 inhibitor, cabazitaxel, organoids, maximum tolerated dose

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1. Introduction

First line therapy for castration resistant prostate cancer (CRPC) involves treatment with androgen receptor (AR) regulators, including abiraterone acetate (ABI), an inhibitor of the androgen synthesis enzyme CYP17A1 and AR inhibitors that bind the AR ligand binding domain (LBD) - including enzalutamide (ENZ), apalutamide (APA) and darolutamide (DARO) (together called androgen receptor signaling inhibitors or ASI). Patients who become refractory to these therapies are subsequently treated with chemotherapeutic agents including cabazitaxel and docetaxel, but resistance to the latter develops quickly as well [1]. Multiple studies indicate that taxane resistance correlates with the expression of AR splice variants [2, 3]. In this project, we hypothesize that (i) ASI treatment promotes Gal-1 expression and nuclear translocation, likely mediated by binding to the splicing factor Gemin4 [4, 5]. We propose that (ii) in the nucleus, Gal-1 cooperates with Gemin4 to promote AR alternate splicing, which induces resistance to ASI and to taxanes. In addition, (iii) Gal-1 has AR independent effects, which allows it to promote growth of AR null tumor cells. However, (iv) inhibition of Gal-1 will suppress alternately spliced AR as well as AR independent cell growth, resulting in growth inhibition of the CRPC cells. The ***specific objective*** of this project is to investigate whether the newly developed Gal-1 inhibitor S-LLS30 and its derivative S-LLS133 sensitize non-responders to this chemotherapeutic agent in AR positive and AR-negative CRPC tumors, and to identify off-target effects. We intend to develop an efficacious gal-1 inhibitor that sensitizes tumor cells to taxanes in post-ABI CRPC, a disease stage that currently lacks any specific treatment.

2. Keywords

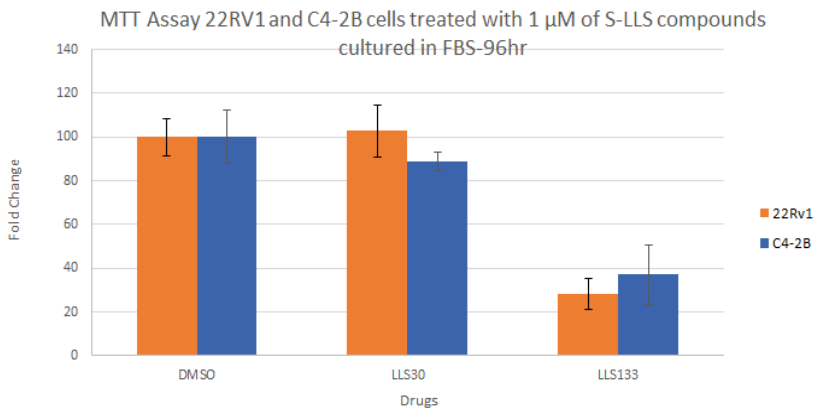
Galectin-1, castration resistant prostate cancer, cabazitaxel, galectin-1 inhibitor, maximum tolerated dose, organoids

3. Accomplishments

a. The major goals of the project and accomplishments under these goals in the reporting period

Major Task 3: To evaluate the toxicity and stability of S-LLS133 in vivo	Months
Subtask 1: Evaluation of toxicity to identify the optimal dose of S-LLS133	24-36

A



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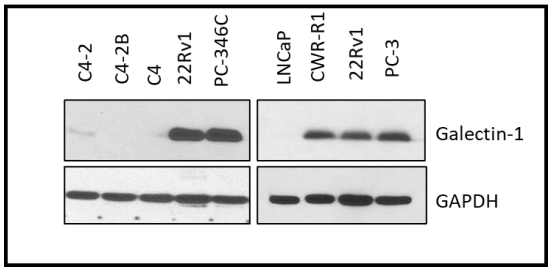


Fig 1. **A**) MTT assay results. **B**) Galectin-1 expression in different prostate cancer cell lines.

Discovery of a novel and improved galectin-1 inhibitor. As reported in last year's report, we recently discovered a novel derivative of S-LLS30 named S-LLS133 which exhibits more potent anti-cancer effect *in vitro* than S-LLS30. It preferentially kills galectin-1 expression 22RV1 cell than C4-2B cell which does not express galectin-1 (**Fig 1A** and **1B**). Preliminary data indicate that it binds to Gal-1 and inhibits proliferation as well as Gal-1 function **(Fig. 2).**

S-LLS133 binds to galectin-1:

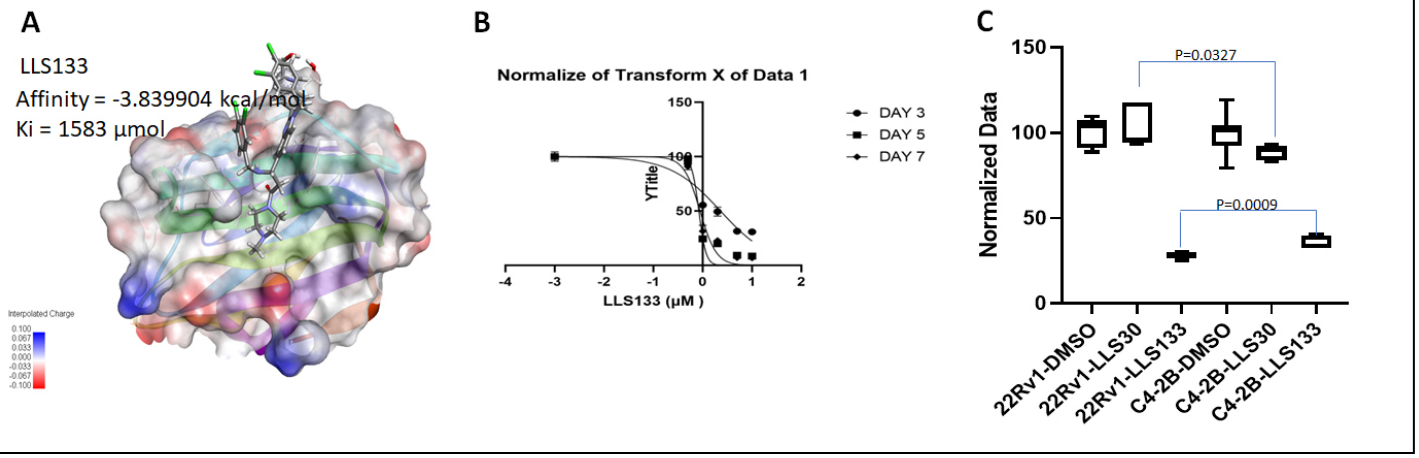


Figure 2: Structure of S-LLS133 showing affinity binding to a major groove of galectin-1. (A) Affinity binding of S-LLS133 to Gal-1. **(B)** Growth curves at different concentration of S-LLS133 in castration resistant C4-2 cells, showing an IC_{50} = 0.5316 µM. **(C)** LGALS1 levels affected by S-LLS133.

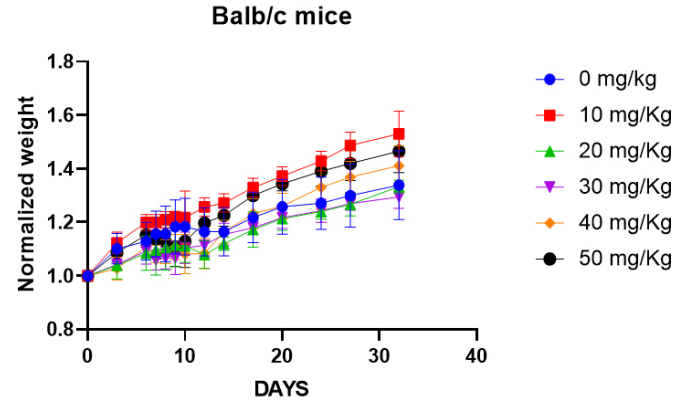


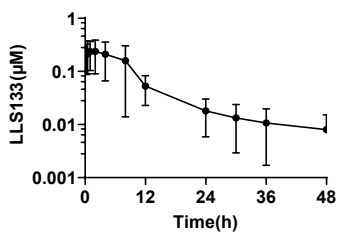
Figure 3. Mouse weight change during MTD study of S-LLS133. The drug was administered at the doses of 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg and 50 mg/kg for 5 days. On day 6, 2 animals/group were submitted to CPL, UC Davis. The rest mice were monitored for 30 more days and euthanized at day 35.

S-LLS133 is not toxic in vivo We performed a Maximum Tolerated Dose (MTD) experiment to identify the highest dose of S-LLS133 that can be safely administered to mice**.** No signs of toxicity were observed in the groups that received S-LLS133 for 5 consecutive days. The animals continued to grow normally, as shown by the gradual increase in animal weight from baseline: (**Figure 3**). At day 35, 30 days after the last dose of S-LLS133, all animals showed weight increase.

Conclusions: Based on these results, S-LLS133 can be safely used in mice.

Subtask 2: *In vivo* metabolic stability study and metabolite identification of S-LLS133 | **34-36**

Pharmacokinetic (PK) studies of S-LLS133. LC-MS/MS based assays was used to define the metabolic stability of S-LLS133. Rats (n=4) was administered S-LLS133 by i.p. at 30 mg/kg for PK study (**Fig 4**). Rat plasma levels of the compounds over 48 h was evaluated by LC-MS/MS assay and stability of the compound determined. The half-life ($t_{1/2}$) of S-LLS133 is 16.2 h, but big variations were seen among rats.



Pharmacokinetic parameters	
T_{max} (h)	1.0±0.7
C_{max} (µM)	0.25±0.14
AUC_{last} (µM*h)	2.3±0.9
T_{half} (h)	16.2±12.1

Figure 4. PK study **of** S-LLS133 in rats (n=4). PK data was processed with Non-Compartmental Analysis, extravascular, Kinetica 5.0.

Conclusions: S-LLS133 is relatively stable in vivo.

Major Task 4: To evaluate the effects of S-LLS133 on the response to cabazitaxel	Months
Subtask 1: To evaluate the effects of S-LLS133 on the response to cabazitaxel	24-30

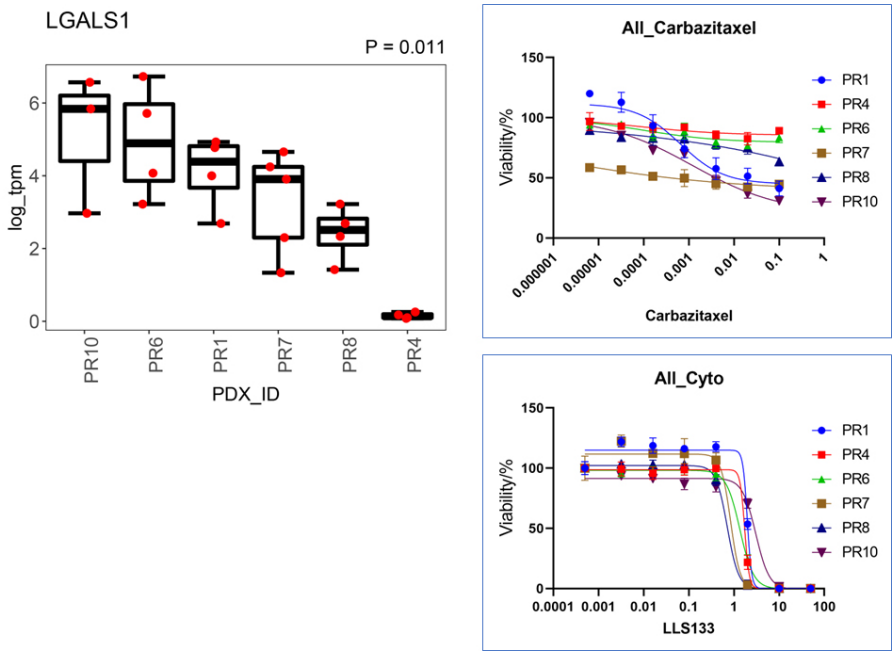


Figure 5. (left) expression of LGALS1, the gene expressing galectin I, in various PDX models of abiraterone resistant prostate cancer. Note that PR10 had the highest level of LGALS1 and PR04 had the lowest. **(right, upper)** response of PDX organoids to the chemotherapeutic agent cabazitaxel in organoids developed from the same mice tumors. Note that PR04 and PR06 are completely resistant to cabazitaxel, while PR07 and PR08 showed only a very small sensitivity. Only PR01 and PR10 showed high responsiveness to cabazitaxel. **(right, lower)** response of PDX organoids to the S-LLS133. Note that all organoids were sensitive to the drug, with an IC_{50} ranging from 0.59 uM for PR08 to 2.87 uM for PR10. PR04 – which has a very low level of LGALS1, still had IC_{50}=1.5 uM for S-LLS133.

As proposed, these studies are being carried out in PDX tumors in immunocompromised mice. However, to identify the best model of the many available, we first tested both cabazitaxel and S-LLS133 in organoids derived from these PDX tumors. **Figure 5** shows that these PDX tumors express different levels of galectin 1, and also responded differentially between the different PDX models. Of these, all but PR04 and PR06 were sensitive to cabazitaxel, and PR08 was extremely sensitive to LLS133 despite expressing low LGALS1. S-LLS133 sensitized cabazitaxel-resistant tumors to this chemotherapeutic agent **(Figure 6)**.

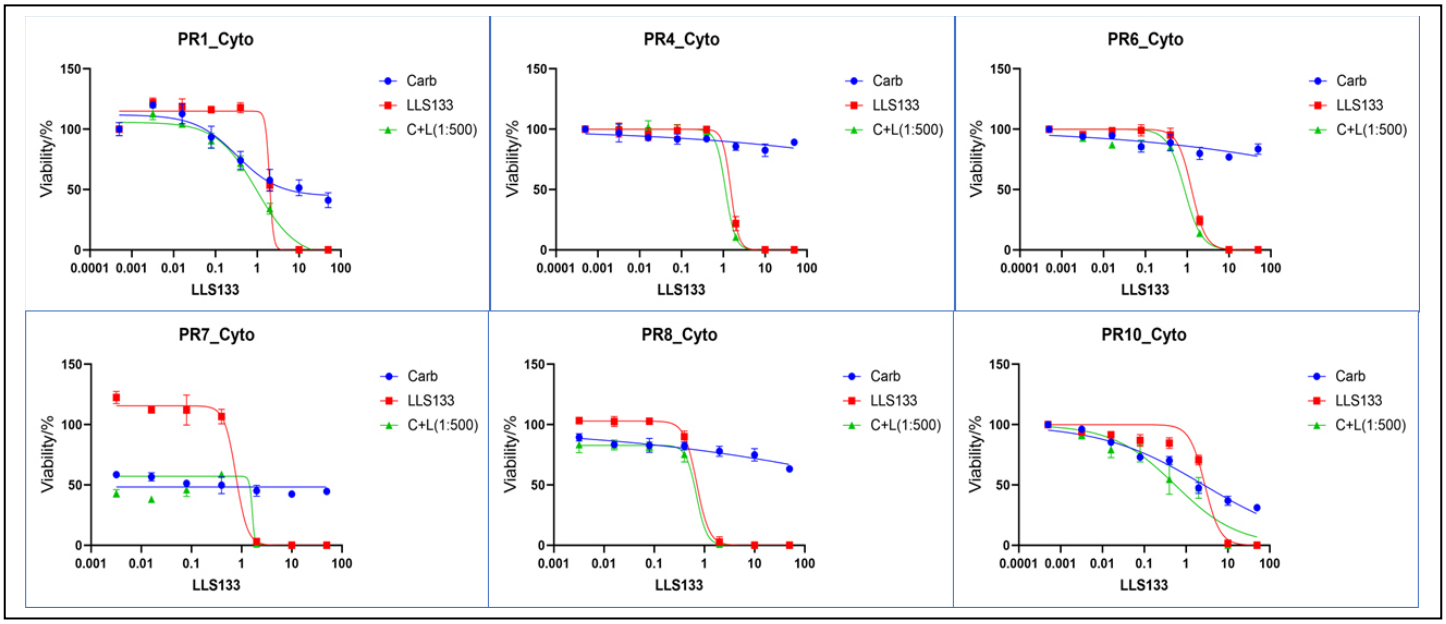


Figure 6: Combination of cabazitaxel and S-LLS133 in different PDX-derived organoids. Results demonstrate that all organoids responded to the combination, including PR04 and PR06 that were resistant to cabazitaxel.

Major Task 5: Determine the effect of S-LLS133 and cabazitaxel on Gal-1 function and	Months

localization	
Subtask 1: Determine whether S-LLS133 is more effective in expressing nuclear or cytosolic Gal-1	**25-30**

Gal-1 expression following S-LLS133 treatment:

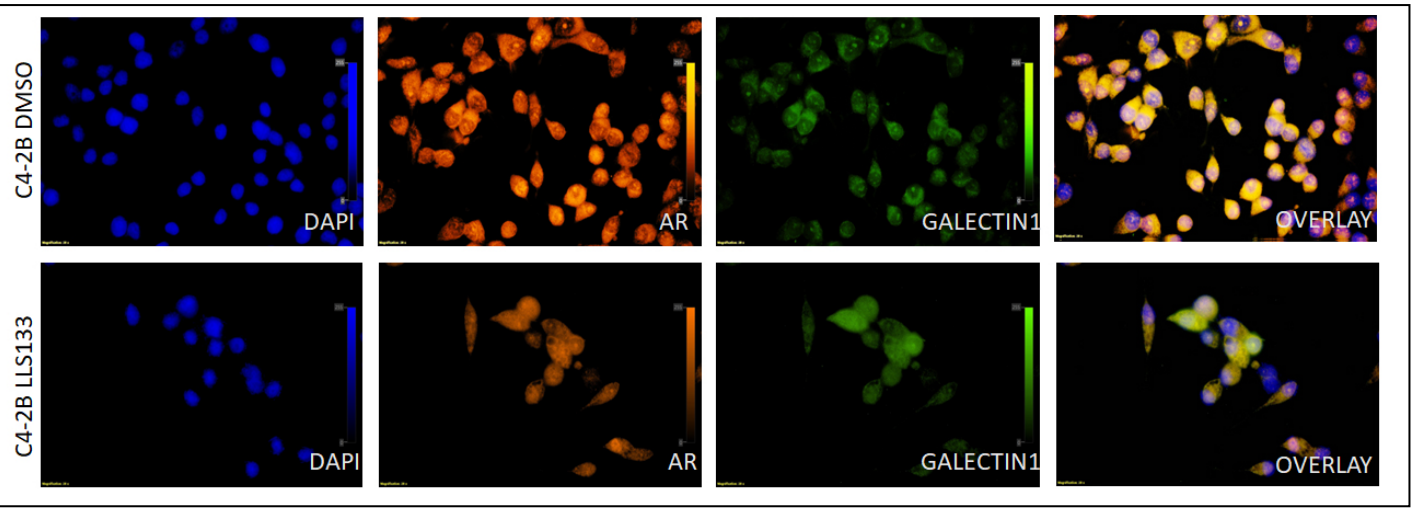


Figure 7A. Expression of Gal-1 in C4-2B cells treated with S-LLS133. This shows that treatment with S-LLS133 enhances nuclear galectin 1.

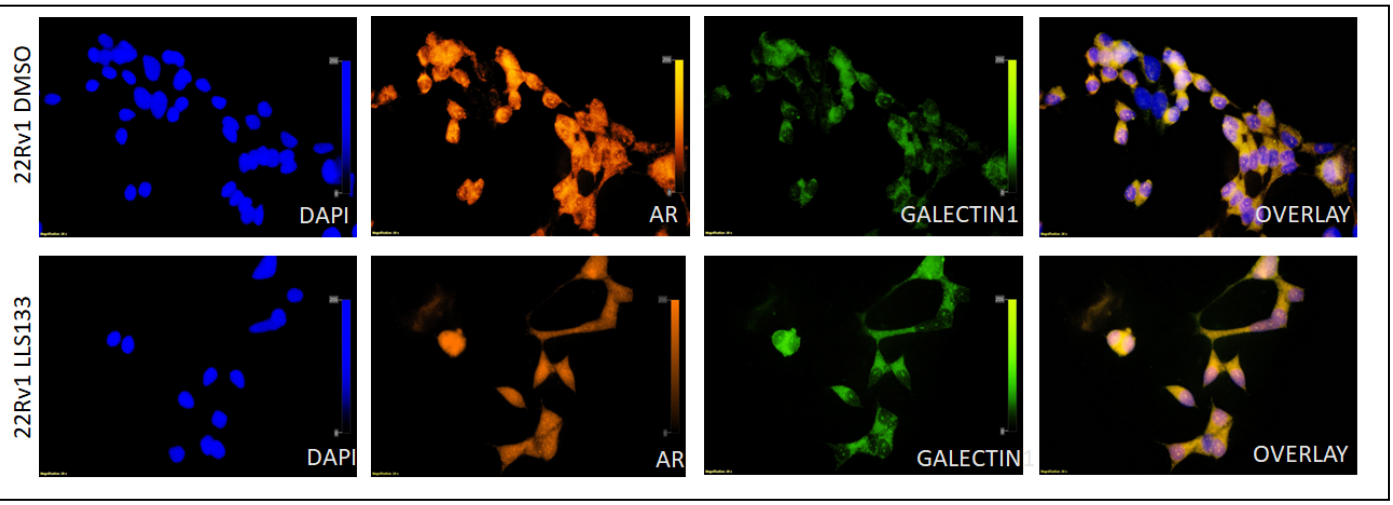


Figure 7B. Expression of Gal-1 in 22Rv1 cells treated with S-LLS133. This shows that treatment with S-LLS133 enhances nuclear galectin 1.

We next investigated whether S-LLS133 affects Gal-1 expression or localization. Figure 7 shows that both C4-2B cells that express low levels of Gal-1 and 22Rv1 cells that express high levels of Gal-1 demonstrated enhanced expression of Gal-1 upon treatment with S-LLS133. Moreover, Gal-1 localized to the nucleus upon treatment with S-LLS133 for 72 hours at 1 μM.

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

Two graduate students were sequentially supported by Year 3 of this award. First, Dr. Maria Malvina Tsamouri, who had already completed her D.V.M., was doing her Ph.D. under the mentorship of Dr. Ghosh from 10/1/2021 – 6/30/2022, when she was supported by this award. After she graduated, a second graduate student, Rebecca Britt Armenta, was associated with this project from 7/1/2022 – 9/14/2022.

c. How were the results disseminated to communities of interest?

Nothing to report. Results have not been disseminated yet**.**

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

In the next funding period, we intend to complete the following:

Major Task 4: To evaluate the effects of S-LLS133 on the response to cabazitaxel of PDX tumors in NSG mice Animal model used: CRPC PDX model (Mayo Clinic, 40 NSG mice) There are four arms of the study (n=10/arm). Mice will be treated with (i) vehicle only, (ii) optimal dose of S-LLS133 (iii) 5 mg/kg cabazitaxel, or (iv) the combination of the two. The treatment will be maintained up to 2 months or until tumor sizes in controls reach 1.0-1.5 cm^3.	37-42		Dr. Wang
Milestone(s) Achieved: The anti-tumor efficacy and toxicity of S-LLS133 in PDX mouse model will be determined.	42		
Major Task 5*:* Determine the effect of S-LLS133 and cabazitaxel on Gal-1 function and localization using the animal study samples from Major Task 4			
Subtask 1: Determine whether S-LLS133 is more effective in PDX expressing nuclear or cytosolic Gal-1 Method: Immunostaining with antibodies to Gal-1 and downstream targets will be used to verify Gal-1 location. Cell proliferation and apoptosis will be determined by Ki67 and cleaved PARP expression, respectively. H-Ras expression by IHC will be used as marker of S-LLS133 activity.	42-45	Dr. Ghosh	
Subtask 2: Determine whether S-LLS133 and/or cabazitaxel affect Gal-1 localization in the tumor. Method: We will compare Gal-1 localization following treatment to that before the treatment using immunohistochemistry.	43-46	Dr. Ghosh	
Subtask 3: **Determine whether localization of Gal-1 and/or its binding partner Gemin4 correlate with the expression of AR splice variants** **Method: Frozen tissue will be used to extract epithelial cells by laser capture micro-dissection and subjected to RNA extraction.** Levels of individual AR variants will be correlated with Gal-1 and Gemin4 localization.	44-48	Dr. Ghosh	
Milestone(s) Achieved: The effect of S-LLS133 and cabazitaxel on Gal-1 function and localization will be determined. Sufficient scientific data will be obtained to determine whether S-LLS133 is a good drug candidate for extensive pre-clinical development.	48		

4. Impact

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

Here, we report on a novel galectin-1 binding molecule developed by the PI called S-LLS133, which is a analog of the galectin-1 inhibitor S-LLS30 developed by the PI earlier. We show that S-LLS133 is far more effective in killing both enzalutamide sensitive C4-2 and enzalutamide resistant 22Rv1 CRPC cells compared to S-LLS30. S-LLS133 has a good binding affinity to the galectin-1 protein. It also has low toxicity and the MTD was not reached in the study in mice, while studies showed that it has high stability. Moreover, using organoids derived from PDX carrying mice, we identify models of CRPC that show high sensitivity and specificity to S-LLS133, despite resistance to cabazitaxel. We show that S-LLS133 affects both cabazitaxel resistant and cabazitaxel sensitive lines, and even sensitized cabazitaxel resistant lines to the drug. Further, our studies demonstrated enhanced galactin-1 localization to the nucleus upon treatment with galectin-1 binding molecule S-LLS133 in both C4-2B cells that express low levels of Gal-1 and 22Rv1

cells that express high levels of Gal-1, therefore suggesting a likely mechanism of action. Taken together, these results suggest that S-LLS133 may be a good drug to target galectin-1 in cabazitaxel-resistant prostate cancer cells.

b. What was the impact on other disciplines?

Nothing to Report.

c. What was the impact on technology transfer?

Nothing to Report.

d. What was the impact on society beyond science and technology?

Nothing to Report.

5. Changes/Problems

Nothing to report.

6. Products

Nothing to report.

7. Participants & Other Collaborating Organizations

Name:	Ruiwu Liu
Project Role:	PI
Researcher Identifier (Credential):	RUIWULIU
Nearest person month worked:	3.0
Contribution to Project:	Dr. Liu oversaw the project and synthesized S-LLS133 for the studies. He worked closely with co-investigators to analyze the experimental results and prepared the progress report.
Funding Support:	

Name:	Paramita M. Ghosh
Project Role:	Co-investigator
Researcher Identifier (Credential):	PAGHOSH
Nearest person month worked:	1.8
Contribution to Project:	Dr. Ghosh oversaw and designed the biological experiments. She has analyzed, interpreted the data and prepared the progress report.
Funding Support:	

Name:	Tsung-Chieh Shih
Project Role:	Co-Investigator
Researcher Identifier (Credential):	TSUNG-CHIEH
Nearest person month worked:	1.2

Contribution to Project:	Dr. Shih assisted PK study.
Funding Support:	

Name:	Maria Malvina Tsamouri
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4.5
Contribution to Project:	In Year 3 of the project, Dr. Maria Malvina Tsamouri, a post-DVM graduate student working on her PhD under Dr. Ghosh, was supported by this award. During Year 1 of the period of performance, Maria Malvina Tsamouri worked on the MTD study for LLS30, but due to an oversight, her contributions were not included in the earlier report. She also trained Rebecca Armenta, M.S. the new graduate student who took over from her, to conduct the animal studies and helped initiate the LLS133 toxicity studies. Malvina also assisted with some in vitro studies for this report.
Funding Support:	During the last funding period, Malvina was funded solely from this project from 10/1/2021 – 6/30/2022. However, at the time she did the MTD studies in Year 1, she was funded by the Maxine Adler and the Lodric Maddox Graduate Student Fellowship Awards from the School of Veterinary Medicine, UC Davis.

Name:	Rebecca Britt Armenta
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.5
Contribution to Project:	Rebecca was responsible for the LLS133 studies reported in Figure 3. She was also responsible for the cell culture associated with the in vitro studies reported here. She was the conduit between the research team and Dr. Salma Siddiqui, who conducted the immunofluorescent studies. She also assisted with the cell culture studies in Figures 1A and 2C. Rebecca was also the conduit between the research team and Dr. Chris Luchhesi who did the affinity binding studies.
Funding Support:	During this period, Rebecca was partly funded by the Pharmacology and Toxicology Graduate Group, University of California Davis.

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

There is no significant change or changes in the level of effort for active support reported previously for the PI and key personnel.

What other organizations were involved as partners?

Organization Name: Mayo Clinic
Location of Organization: 200 First Street SW, Rochester, MN
Partner's contribution to the project: Dr. Liewei Wang (subaward PI) and Dr. Huanyao Gao performed the PDX organoids study and provided documents for HRPO review and approval.

8. **Special Reporting Requirements**

Award chart is attached as appendix.

9. **References**

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Award Log Number: PC180437

Award Title: A Novel Class of Galectin-1 Inhibitors for Prostate Cancer Therapy

PI: Ruiwu Liu, The Regents of the University of California Davis, California **Budget:** $915,156

Topic Area: Prostate Cancer Research Program **Mechanism:** W81XWH-18-PCRP-IDA

Research Area(s): Targeted therapy (0805), Drug resistance (0804) **Award Status: 09/15/21-09/14/22**

Study Goals:

- Determine whether the novel inhibitor S-LLS133 promotes cabazitaxel sensitivity.
- Determine whether S-LLS133 promotes cabazitaxel sensitivity through a mechanism involving galectin-1 (Gal-1) interaction with Gemin4/HSP90.
- Determine whether S-LLS133 is a promising drug candidate for further pre-clinical development.

Specific Aims:

- Aim 1: To determine the mechanism of Gal-1 involvement in cabazitaxel resistance and a potential role for S-LLS133 in overcoming such resistance.
- Aim 2: To test the differential role of nuclear and cytoplasmic Gal-1 in mediating the effects of S-LLS133 on the response of ABI-resistant CRPC to cabazitaxel in patient derived xenografts (PDX) tumors.

Key Accomplishments and Outcomes:

- Treatment with S-LLS133 promote Gal-1 expression and nuclear localization.
- S-LLS133 sensitized cabazitaxel-resistant tumors to this chemotherapeutic agent in organoids derived from abiraterone-resistant prostate cancer PDX tumors expressing different levels of Gal-1.

Publications: none to date

Patents: none to date

Funding Obtained: none to date