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PRINCIPAL INVESTIGATOR: Ruiwu Liu, Ph.D.

CONTRACTING ORGANIZATION: The Regents of the University of California Davis

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14. ABSTRACT
The overall goal of this award is to find ways to prolong the efficacy of cabazitaxel chemotherapy in patients with castration resistant prostate cancer (CRPC) who have previously been treated with and developed resistance to Abiraterone Acetate (ABI) or enzalutamide (ENZ). In months 1-12 of this award, we aimed to determine whether a novel galectin-1 (Gal-1) inhibitor, S-LLS30 developed by the applicant, prevents ABI/ENZ resistance and/or sensitizes the cells to cabazitaxel (Major task 1). We have shown that indeed S-LLS30 sensitizes CRPC cells to ENZ and strongly affected cells expressing Gal-1. The experiments with cabazitaxel are continuing despite prolonged operational shutdown at the University due to COVID-19 restrictions. We have also started to investigate the role of Gal-1 nuclear localization, and its binding partners Gemin4 and HSP90 in this process (Major task 2, subtask 1). It appears that Gemin4 plays a substantial role in Gal-1 activity in this context but the role of HSP90 is unclear. Finally, we conducted preliminary experiments to evaluate the toxicity of S-LLS30 and determine the maximum tolerated dose (Major task 3, subtask 1). S-LLS30 was deemed to be of limited toxicity and very well tolerated in mice up to 30 mg/Kg doses. S-LLS30 is a viable potential drug candidate to overcome resistance to ABI/ENZ in models of CRPC.

15. SUBJECT TERMS
Galectin-1, castration resistant prostate cancer, enzalutamide, galectin-1 inhibitor, gemin4, androgen receptor, cabazitaxel

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TABLE OF CONTENTS

1. Introduction

During abiraterone acetate (ABI) and enzalutamide (ENZ) treatment (together called androgen receptor signaling inhibitors or ASI) as first line therapy for castration resistant prostate cancer (CRPC), some patients become refractory to these therapies and are subsequently treated with cabazitaxel, but resistance to the latter chemotherapeutic agent develops quickly as well [1]. Multiple studies indicate that taxane resistance correlates with the expression of AR splice variants [2, 3]. Here, we hypothesize that (i) ASI treatment promotes Gal-1 nuclear translocation likely bounds to the splicing factor Gemin4, as suggested by previous reports [4, 5]. We propose that (ii) in the nucleus, Gal-1 cooperates with Gemin4 to promote AR expression and alternate splicing, which induces resistance to ASI and to docetaxel. Hence, (iii) inhibition of Gal-1 suppresses levels of both full-length and alternately spliced AR, which causes growth inhibition in AR positive cells. However, (iv) Gal-1 has AR independent effects as well, which allows it to promote growth of AR null tumor cells. The ***specific objective*** of this project is to investigate whether the newly developed Gal-1 inhibitor S-LLS30 sensitizes non-responders of cabazitaxel to this chemotherapeutic agent in AR positive and AR-negative CRPC tumors, and to identify off-target effects of S-LLS30. We intend to develop S-LLS30 into an efficacious drug 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, enzalutamide, galectin-1 inhibitor, gemin4, maximum tolerated dose, androgen receptor

3. Accomplishments

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

Major Task 1: To determine whether S-LLS30 promotes cabazitaxel sensitivity by preventing ABI/ENZ-induced Gal-1 nuclear localization	Months
Subtask 1: To synthesize and purify S-LLS30 (completed)	1-3
An improved synthetic approach has been developed to avoid using expensive and toxic chemical reagents. More than 2g of S-LLS30 has been synthesized and purified with high purity (>98%).	
Subtask 2: To investigate whether ABI or ENZ treatment induces nuclear localization of Gal-1, and S-LLS30 reverses this effect (50% completed)	4-12

S-LLS30, but not ENZ, induces cytoplasmic localization of Gal-1. 22Rv1 cells were treated with DMSO (vehicle), 2 μM ENZ or 1.2 μM S-LLS30 for 48 hours and stained with antibodies to Gal-1 or α-tubulin by immunofluorescence. DAPI staining was used to identify the nucleus. Rhodamine-tagged α-tubulin is primarily cytoplasmic and therefore is a marker of cytoplasmic proteins. FITC-tagged Gal-1 is observed both in the nucleus and the cytoplasm in DMSO treated cells. Cytoplasm localization is shown by yellow color resulting from overlap of green (Gal-1) and red (α-tubulin) stains, indicating co-localization. Note that some cytoplasmic Gal-1 is observed in DMSO-treated cells, but decreases substantially with ENZ and is reinforced significantly with S-LLS30 treatment.

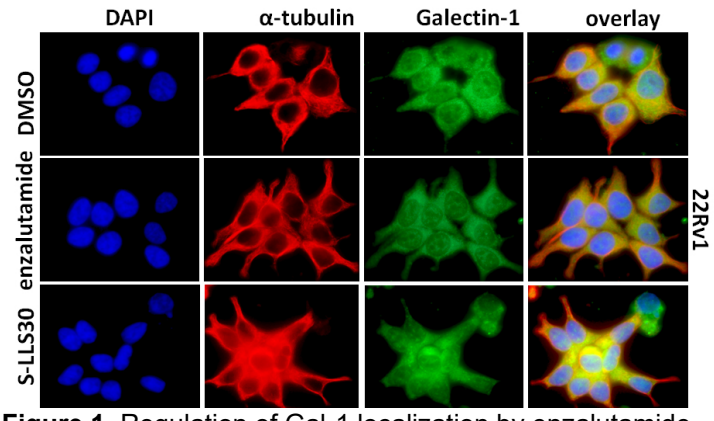

Figure 1. Regulation of Gal-1 localization by enzalutamide and S-LLS30. DAPI staining denotes location of nucleus.

Ongoing work: We are currently conducting western blots and ELISA as planned to verify these results.

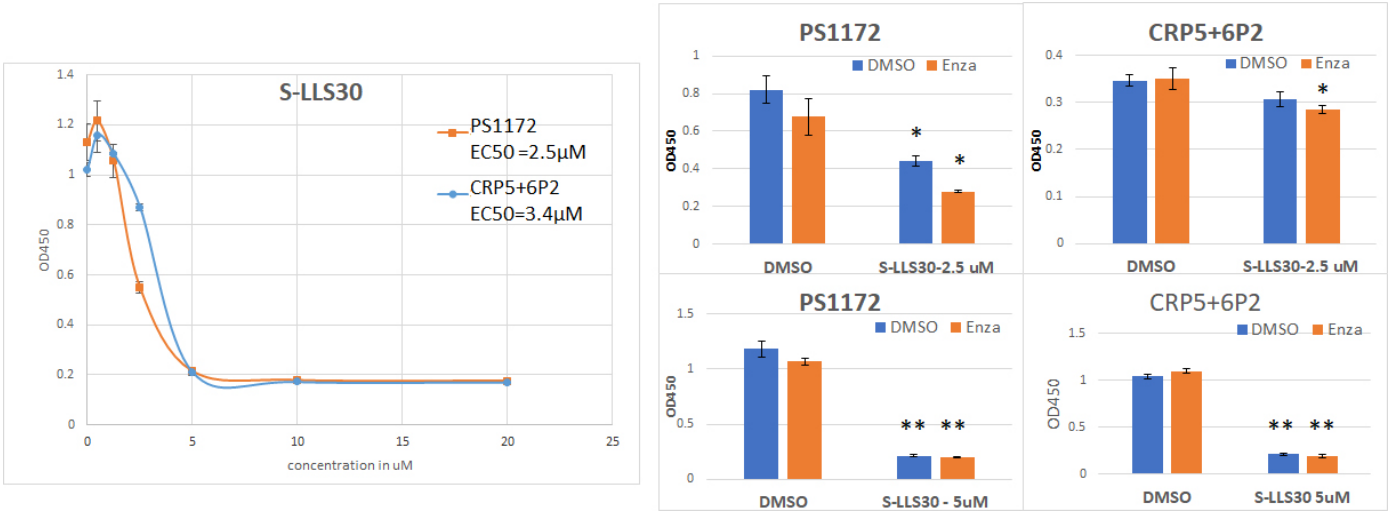

Figure 2. Response of cells from a patient derived xenograft (PDX) model of prostate cancer progression on enzalutamide to S-LLS30.

Establishment of cell line models of enzalutamide resistant prostate cancer that responds to S-LLS30. We tested whether S-LLS30 induced sensitivity to enzalutamide in PS1172 and CRP5+6P2 cell lines. PS1172 is an established cell line from a previously developed patient derived xenograft (PDX), which has high fidelity to the original tumor and is somewhat sensitive to enzalutamide. An enzalutamide resistant cell line CRP5+6P2 was further developed from PS1172. **Figure 2** shows that both PS1172 and CRP5+6P2 lines were sensitive to S-LLS30 with an EC_{50} of 2.5 µM and 3.4 µM in PS1172 and CRP5+6P2 cells, respectively. Based on these results, we further tested whether S-LLS30 sensitized these lines to enzalutamide. At 2.5 µM, S-LLS30 had a significant effect on PS1172 and sensitized the cells to enzalutamide, but the effect in CRP5+6P2 was less distinct. On the other hand, at 5 µM, both PS1172 and CRP5+6P2 responded to S-LLS30 alone, enzalutamide did not have any additional effect.

Ongoing Studies: In future studies, we will use the models to test the effect of S-LLS30 on cabazitaxel resistance. These cell lines were not available to us at the time of grant application, but they are worthwhile models to pursue for future studies.

Subtask 3: To prepare animal use protocols for review and approval (completed)	1-4

We have submitted and received approval for animal use. Both IACUC approval from UC Davis and ACURO approval from the DoD has been received. In addition, IACUC protocol has been approved for efficacy study using established prostate PDX models in Mayo Clinic. DoD ACURO and HRPO regulatory review and approval are complete.

Major Task 2: To determine whether S-LLS30 promotes cabazitaxel sensitivity by preventing ABI-induced Gal-1 nuclear localization through a mechanism involving Gal-1 interaction with Gemin4/HSP90.	Months
Subtask 1: Determine the role of Gemin4 and HSP90 in mediating Gal-1 nuclear translocation (50% completed)	10-14

Both Gal-1 and GEMIN4 expression is suppressed by culture of cells in charcoal stripped serum. As Gal-1 has not been identified to have a nuclear localization signal (NLS), we hypothesized that it piggybacks into the nucleus with its established binding partner Gemin4. We have shown in **Figure 1** that AR inhibition by enzalutamide caused a decrease in cytoplasmic Gal-1, hence we surmised that AR inhibition will increase Gal-1 transportation out of the nucleus. To determine whether Gemin4 indeed plays a role in Gal-1 nuclear translocation and whether this action is influenced by AR inhibition, we compared Gal-1 and Gemin4 expression in regular medium containing FBS (which expresses high levels of androgens) and in CSS containing low androgens. **Figure 3** shows that androgen deprivation decreased the levels of both Gal-1 and Gemin4 but the effect on Gemin4 is more, and Gemin4 expression was significantly decreased in the absence of AR activity.

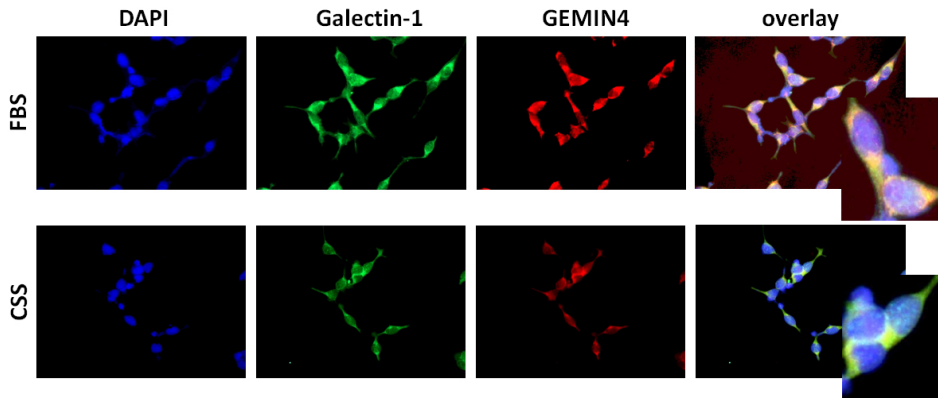

Figure 3. Expression of Gal-1 and its binding partner GEMIN4 in regular medium with fetal bovine serum (FBS) and in androgen-deprived medium with charcoal stripped serum (CSS). DAPI staining denotes location of nucleus. Magnification – 20X. Inset – enlarged overlay showing co-localization of Gal-1 and Gemin4 in FBS, but lack of Gemin4 in CSS.

Ongoing work: We are currently conducting western blots and co-immunoprecipitation of Gal-1 with Gemin4 and HSP90 as planned.

Major Task 3: To evaluate the toxicity and stability of S-LLS30 *in vivo*	Months
Subtask 1: Evaluation of toxicity to identify the optimal dose of S-LLS30 (completed)	**7-12**

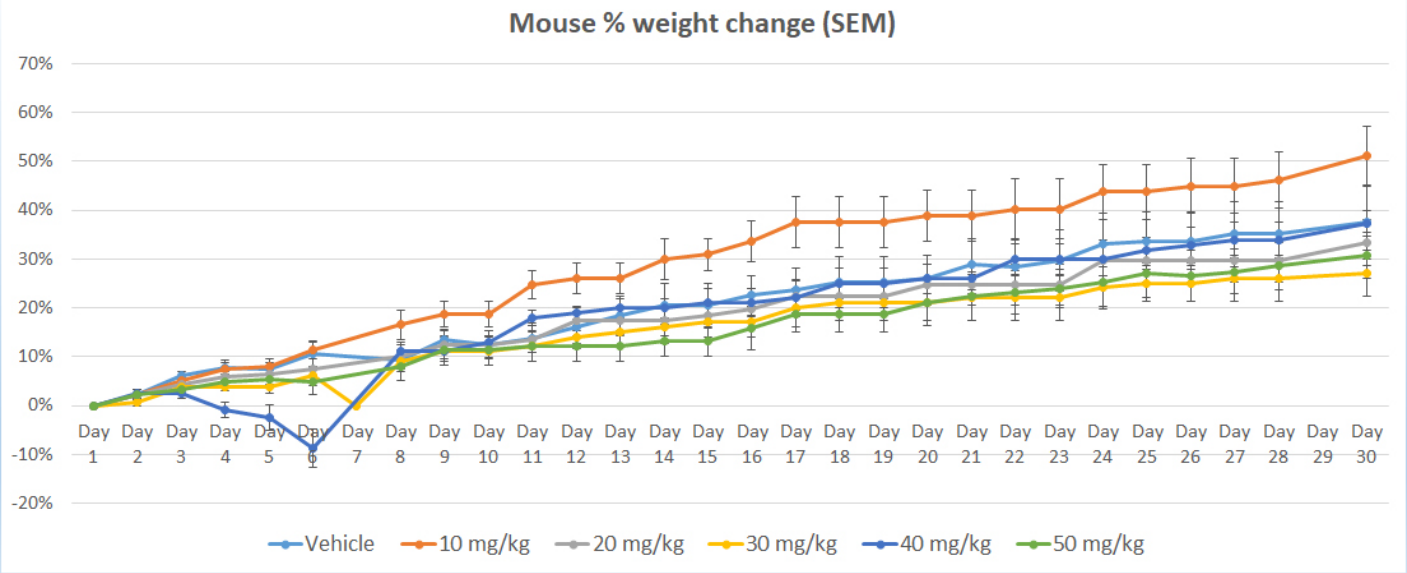

Figure 4. Mouse weight change during MTD study of S-LLS30. S-LLS30 was administered at the doses of 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg and 50 mg/kg for 5 days. At day 6, 4 animals/group were submitted to CPL, UC Davis. The rest mice were monitored for 30 more days and euthanized at day 35.

30 mg/kg was established as the MTD of S-LLS30 in vivo We performed a Maximum Tolerated Dose (MTD) experiment to identify the highest dose of S-LLS30 that can be safely administered to mice**.** Some animals showed a transient discomfort, due to minor bleeding after the injections. No signs of toxicity were observed in the groups that received Control, 10 mg/kg, 20 mg/kg and 30 mg/kg for 5 consecutive days. The animals

continued to grow normally, as shown by the gradual increase in animal weight from baseline: mean=11%, SEM=1% for control group and mean= 8%, SEM=2%, mean=6% SEM=1%, mean=4%, SEM=1%, mean=5%, SEM=1%, increase from baseline at day 5 for 10 mg/kg, 20 mg/kg, 30 mg/kg and 50 mg/kg S-LLS30-treated groups respectively (**Figure 4**). At day 35, 30 days after the last dose of S-LLS30, control animals showed a mean= 43% weight increase (SEM=8%), 10 mg/kg treated animals a mean= 55% weight increase (SEM=6%), 20 mg/kg treated animals showed a mean body weight increase of 36% (SEM=6%), 30 mg/kg treated animals a mean body weight increase of 30% (mean=4%) and finally, 50 mg/kg treated animals showed a mean body weight increase of 34% (SEM=6%).

However, the 40 mg/kg group, showed a 2% weight reduction from baseline at day 5 (SEM= 3%), when compared to the control group (**Figure 4**). Four out of 8 animals showed a weight reduction of 14%, 5%, 5% and 10%, whereas the rest continued to grow normally. As said, before, none of the animals administered the dose of 50 mg/kg showed any weight reduction. However, since at least one animal met the endpoint of 10% weight reduction, the dose of 40 mg/kg was considered non-tolerated and 30 mg/kg was established as the MTD. These animals continued to be monitored for 30 days and it should be noted that they quickly recovered after the withdrawal of the drug, showing a mean body weight increase of 46% (SEM=3%) at day 35.

Blood chemistry

Kidney function was evaluated through blood urea nitrogen (BUN) and creatinine levels. BUN was within the normal range for the species for all animals (15.2-34.mg/dL). 50 mg/kg treated animals showed a slight increase in creatinine levels at day 6 (mean= 0.52, SD=0.31, whereas the values for rest of the groups fell between the normal range (0-0.3 mg/dL).

Liver function was assessed through Alkaline phosphatase (ALP), Alanine transaminase (ALT), Aspartate transaminase (AST) as well as by albumin, total protein and total bilirubin levels. ALT, AST, Albumin, total bilirubin and total protein levels were withing the normal range for Balb/c mice. AST was increased in 50 mg/kg S-LLS30 treated group, however it was not diagnostic due to the presence of hemolysis that can falsely elevate ALP. However, all groups showed an increase in ALP levels (mean=302.7, SD=1.32 for the control group, mean= 338.27, SD= 1.12 for 10 mg/kg treated group, mean= 265.43, SD= 46.53 for 20 mg/kg treated group, mean= 298.9, SD= 7.71 for 30 mg/kg treated group, mean= 290.40, SD= 25.13 for 40 mg/kg treated group and mean=264.13, SD= 101.36 for 50 mg/kg treated group , normal range= 49-172 U/L. The fact that this liver toxicity was present in all groups, including the control that only received vehicle indicates that either the animals could not tolerate the vehicle itself (8.7% Tween 80 and 8.7% Ethanol in PBS) or that there was a pre-existing issue with these animals. In any case, this toxicity cannot be attributed to LLS30, as the control animals did not receive any of the drug.

Blood hematology

The number of white blood cells (WBC), Absolute neutrophils, lymphocytes, monocytes and basophiles were within the normal range for the species. There was a slight increase in absolute eosinophils in the 10 mg/kg treated group (mean=0.12, SD= 0.06, normal range = 0-0.1K/UL) but the rest of the groups showed normal levels. Lymphocyte % and monocyte % were within the normal range whereas there was a slight increase in neutrophils % in 20 mg/kg treated group (mean= 34.15, SD= 5.85, normal range= 12.5-31.2%). 10 mg/kg treated group showed a mild increase in eosinophil (mean= 1.25, SD= 0.62, normal range = 0-0.8%), basophils (mean= 0.58, SD= 0.27, normal range= 0-0.5%), Read blood cells (RBC, mean= 11.29, SD= 0.81, normal range= 8.12-10.55 M/uL). Hemoglobin (mean=17, SD= 1.37, normal range= 11.7-16.2 g/ dL) and hematocrit (mean= 56.83, SD= 4.48, normal range= 38.3-54). 50 mg/kg treated group showed a slight increase in eosinophils (mean=0.86, SD= 0.33, normal range= 0-0.8%), basophils (mean=0.62, SD= 0.37, normal range= 0-0.5%), RBC (mean= 11.46, SD= 0.23, normal range=8.12-10.55 M/uL), hemoglobin (mean= 17.56, SD= 0.47, normal range= 11.7-16.2 g/dL), and hematocrit (mean=58.43, SD= 1.60, normal range= 38.3-54%). Finally, the absolute number of platelets was increased in the control (mean=1261, SD= 109.88), 20 mg/kg (mean= 1099.67, SD= 128.40), 30 mg/kg (1215.67, SD= 222.26) and 40 mg/kg (mean=1259, SD=125.03) treated groups (normal range for platelets=574-1079 K/uL).

Conclusions: Based on these results, S-LLS30 can be safely used in rodents at 30 mg/Kg.

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

We have had two trainees on this project. Thomas M. Steele was a Junior Specialist who had graduated with a BS degree from UC Davis. Upon completion of his training, he is now applying to medical school and decided

to separate from the project. In his place, we hired Dontrel Hairston, a graduate student with the Pharmacology and Toxicology Graduate Group in UC Davis. Dontrel has just started his training.

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 1: To determine whether S-LLS30 promotes cabazitaxel sensitivity by preventing ABI/ENZ-induced Gal-1 nuclear localization	Months
Subtask 2: To investigate whether ABI or ENZ treatment induces nuclear localization of Gal-1, and S-LLS30 reverses this effect (contd). Method: The cells will be subjected to subcellular fractionation into nuclear and cytoplasmic fractions using Western blotting, and Gal-1 levels will be determined by Western blotting. Secreted Gal-1 will be evaluated by ELISA. The effect of AR expression, mutation and androgen withdrawal on Gal-1 localization will be investigated by western blotting. As stated below, this subtask would have been completed but could not due to lab shutdown due to COVID-19 concerns. The work will be resumed in the next funding period.	10-14
Major Task 2: To determine whether S-LLS30 promotes cabazitaxel sensitivity by preventing ABI-induced Gal-1 nuclear localization through a mechanism involving Gal-1 interaction with Gemin4/HSP90.	Months
Subtask 1: Determine the role of Gemin4 and HSP90 in mediating Gal-1 nuclear translocation (contd). Method: 22Rv1 and CWR-R1 cells will be cultured in charcoal stripped serum for 72 h to remove androgens and then treated with increasing doses of dihydrotestosterone (DHT). Gal-1 binding to Gemin4 and HSP90 will be determined by co-immuno precipitation, and other heat shock proteins (HSP70, HSP27) will be identified by immunoblotting. Amount of AR bound to HSP90 will also be similarly estimated. Localization of each protein will be tested by Western blotting in subcellular fractionated cells. Cells will be treated with increasing doses of ABI/ENZ and binding studies conducted.	10-14
Subtask 2: Determine whether S-LLS30 prevents Gal-1/Gemin4 nuclear translocation required for AR alternate splicing. Method: Gal-1 in 22Rv1 and CWR-R1 cells and AR splice variant expression will be determined by qPCR. The cells will be treated with S-LLS30 and the effect on Gal-1 and Gemin4 nuclear localization will be determined in the presence and absence of the drug using subcellular fractionation and by immunofluorescence.	15-18
Major Task 3: To evaluate the toxicity and stability of S-LLS30 *in vivo*	Months
Subtask 2: *In vivo* metabolic stability study and metabolite identification 12 rats implanted with Jugular vein catheter will be used.	13-24

4. **Impact**

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

Our studies demonstrated that S-LLS30 was safe to use up to 30 mg/Kg dose. Further, we show that Gemin4, the binding partner of Gal-1 – may be androgen regulated. We have established novel cell line models of prostate cancer progression that can be regulated by S-LLS30. Finally, we establish that S-LLS30 regulates gal-1 localization.

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**

We are on track to complete all the projects as projected, however, due to COVID-19, our labs were shut down completely from the third week of March to the end of May and is still partially restricted due to California state laws dictating how many people can be in the labs at the same time. As a result, we are about 3 months behind on some of our tasks.

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-LLS30 for the studies. He worked closely with co-investigators to analyze the experimental results.
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 and interpreted the data.
Funding Support:	

Name:	Maria Malvina Tsamouri
Project Role:	Graduate Student
Researcher Identifier (Credential):	
Nearest person month worked:	1.0
Contribution to Project:	Maria Malvina Tsamouri worked on the MTD study for S-LLS30.

Funding Support:	Maria Malvina Tsamouri was funded by the Maxine Adler and the Lodric Maddox Graduate Student Fellowship Awards from the School of Veterinary Medicine, UC Davis

Name:	Tsung-Chieh Shih
Project Role:	Co-Investigator
Researcher Identifier (Credential):	TSUNG-CHIEH
Nearest person month worked:	1.2
Contribution to Project:	Dr. Shih provided advice on the design of MTD and toxicity studies.
Funding Support:	

Name:	Thomas M. Steele
Project Role:	Assistant Specialist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.7
Contribution to Project:	Mr. Steele has assisted with all the immunofluorescence studies reported in this progress report.
Funding Support:	

Name:	Dontrel Hairston
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.4
Contribution to Project:	Dontrel conducted and is currently continuing to conduct western blots pertaining to this project.
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?

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

8. **Special Reporting Requirements**

Award chart is attached as appendix.

9. References

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