AD_____

AWARD NUMBER: W81XWH-14-1-0552

TITLE: Targeting Siah2 as Novel Therapy for Metastatic Prostate Cancer

PRINCIPAL INVESTIGATOR: Neil Bhowmick

RECIPIENT: Cedars-Sinai Medical Center
Los Angeles, CA 21702-5014

REPORT DATE: October 2016

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sep 2015 – 29 Sep 2016

4. TITLE AND SUBTITLE

Targeting Siah2 as Novel Therapy for Metastatic Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0552

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Neil Bhowmick

E-Mail: ronai@sbpdiscovery.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Cedars-Sinai Medical Center
Los Angeles, CA 21702-5014

8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel 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 goal of this project is to develop a novel means to inhibit prostate cancer development and progression. The development of Siah1/2 inhibitors to the ubiquitin ligase Siah1/2 has been advanced by the ability to develop a Siah1/2 inhibitory peptide that effectively inhibits Siah1/2 activity, which was found to effectively attenuate the growth of prostate cancer tumors *in vivo* when transplanted subcutaneously or orthotopically into the prostate site. The assessment of the Siah1/2 inhibitory peptide in genetic models of mouse as in human PDX tumors is ongoing by the Partnering PIs Drs. Martin Gleave and Neil Bhowmick at the two respective sites. Parallel development of small molecule inhibitors to Siah2 is on going and will complement the work performed with the inhibitory peptide. This is a first-in-class inhibitor of the ubiquitin ligase that can be administered intravenously and that has a notable effect on prostate cancer growth *in vivo*.

15. SUBJECT TERMS

Prostate cancer (PCa); castration resistant PCa; neuroendocrine PCa; Siah1/2; ubiquitin ligases; androgen receptor; HIF1α

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	27	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

1. Introduction 1

2. Keywords 2

3. Accomplishments 2

4. Impact 9

5. Changes/Problems 10

6. Products 11

7. Participants & Other Collaborating Organizations 12

8. Special Reporting Requirements 18

9. Appendix 18

1. INTRODUCTION

Being the most diagnosed malignancy in men and the second leading death of cancer-related diseases, prostate cancer (PCa) remains a significant clinical challenge (Wyatt and Gleave, 2015). PCa initially responds to the first line androgen deprivation therapy (ADT) or androgen receptor (AR) pathway inhibition (ARPI) but eventually develops into lethal castration resistance prostate cancer (CRPC, Loriot et al., 2012). The most recognized AR-negative CRPC variant is neuroendocrine PCa (NEPC), which is characterized by the expression of neuroendocrine markers such as chromogranin A (CHGA), synaptophysin (SYP) and neuron-specific enolase (NSE). NEPC is highly aggressive and poorly diagnosed, and the mechanisms underlining trans-differentiation of NEPC remain elusive. Therapeutic targeting of NEPC is challenging due in part to their aggressiveness and similarity to neuronal cells. There is an urgent unmet need for mechanistic understanding and novel therapy candidates for this lethal disease variant (Toren and Gleave, 2013).

Among those mechanisms being tested, the Siah2 protein has shown significant support on the progression of CRPC (Qi et al., 2013) and NEPC (Qi et al., 2010). Playing the role of an ubiquitin E3 ligase, Siah2 selectively triggers degradation of a subset pool of inactive AR therefore promoting expression of a sub-pool of AR target genes (Qi et al., 2013). Siah2 also facilitates the ubiquitination and degradation of prolyl hydroxylase 3 (PHD3), hence allows stabilization of the HIF1α protein (Nakayama et al., 2004) and modulates the expression of HIF1α-associated genes (Qi et al., 2010; Nakayama et al., 2004). Furthermore, Siah2 regulates the tight junction integrity and cell polarity under hypoxia conditions by modulating availability of protein ASPP2 (Kim et al., 2014). Siah2 is markedly increased in CRPC and Siah2 inhibition promotes prostate cancer regression upon castration (Qi et al., 2013). Therefore, Siah2 has become a promising therapeutic target for CRPC and NEPC (Qi et al., 2013; Qi et al., 2010).

In the course of the first year it became apparent that the lead inhibitor (SBI-852) may not have sufficient biophysical properties to mediate the anticipated activities *in vivo*. We have thus, begun, as reported in the first year's progress report, to screen for alternate small molecules and further extended ongoing studies for the development of peptide inhibitors for Siah ubiquitin ligases.

The second year has been devoted to further advance the development of new Siah small molecule inhibitors and specific Siah peptide inhibitors, while evaluating some of the more recent inhibitors developed in the course of the first year. We have made significant progress on both fronts, as noted below.

We have completed two new high-throughput screens for Siah inhibitors. First, we used the thermal shift assay to screen for Siah bound small molecules that will alter its melting temperature. Out of 32,000 small molecules, we identified 80 that affect the melting temperature, of which 15 were chosen for further assessment in rigorous biochemical and cell biological assays. Those include assessment for the small molecule effect on Siah ubiquitination, as well as the effect on the stability of Siah substrates, HIF1α and ERK phosphorylation (which are regulated by Siah control of PHD1/3 and Sprouty2, respectively). We found that three compounds exhibit promising properties, although they failed to show rigorous effect in a second layer of assays that require competition of Siah substrates. Thus, we performed a modified screen that used a full-length, purified form of Siah1 (the earlier screen used a truncated form of the protein given the technical difficulties to produce full length protein). It required cloning into several expression vectors to produce in insect the full-length protein, which was affinity purified over a 4-month period to enable a small-scale screen for Siah inhibitors. This second screen was based on the principles of the first one, where we assessed the melting temperature changes by any of the 80 small molecules identified in the first screen, and further, added a new library of 500 defined inhibitors to this screen. Of 12 hits, we selected one with superior properties as a promising new inhibitor for Siah. This single compound exhibits the ability to inhibit

Siah self ubiquitination *in vitro* (using purified reagents) and *in vivo* (using cell based assays). It also effectively attenuates the stability of HIF1α, as expected from a Siah inhibitory protein. We have now designed and are about to test ~20 derivatives, analogues for this newly identified small molecule, where we will determine if we are able to identify the critical domain required for its activity, and further improve on its biophysical properties. Parallel work is carried out with the newly selected small molecule inhibitor of Siah to determine its effectiveness in inhibiting prostate cancer cells in culture, where initial experiments hold great promise. Ongoing are initial *in vivo* studies using the human prostate cancer xenografts, CW22RV1 and PC3 cells. We expect that work done within the second year will be completed over the next few months, and will allow us to define the more effective small molecule that inhibits Siah and test it in a series of mouse and PDX models using the models available to Drs. Gleave and Bhowmick. To this end, we have been using four *in vivo* models to evaluate the impact of these inhibitors on PCa development and progression, with a focus on CRPC and NEPC.

The model assessed by Dr. Gleave, relies on the Shionogi mouse model, a mouse androgen-dependent mammary carcinoma that, like human prostate cancer, regresses after castration and later recurs as an androgen-independent tumor. In this model, androgen-dependent tumors in intact mice undergo complete regression following androgen ablation, but rapidly growing androgen-independent tumors recur after 1 month in a highly reproducible manner (Bruchovsky et al., 1990). Therefore, this model is particularly useful to evaluate the efficacy of agents targeting progression to androgen independence (Miayake et al., 2000). The second *in vivo* model, which is also assessed in Dr. Gleave's laboratory, is the patient derived PDX model, which is highly clinical relevant (Lin et al., 2014). The PDX LTL352 model was developed from a patient's metastatic poorly differentiated NEPC. When grafted under the renal capsules of NOD-SCID mice, LTL352 xenografts show androgen-independent tumor growth and invade into adjacent host kidney parenchyma and metastases to distant organs. It expresses typical neuroendocrine markers (e.g., CHGA and SYP with absence of expression of AR). Another PDX model LTL331 was derived from hormonal naive prostatic adenocarcinoma tissue and retained key properties of the original tumor, including histopathological, genomic and transcriptomic characteristics. Castration of mice carrying LTL331 tumors leads to a decrease in tumor volume and plasma PSA levels. However, tumors recur after 6–8 months. This recurred tumor line, designated LTL331R (which will be used in this study) is highly proliferative and showed androgen-independent growth. It was entirely AR and PSA negative, uniformly expressed a range of neuroendocrine markers, including SYP, CHGA, CHGB and CD56. Importantly, recent clinical follow-up information showed that the patient, from whom the LTL331 line had been derived, developed NEPC after long-term androgen ablation therapy. The third tumor model was evaluated by Dr. Bhowmick. This model utilizes the Beige-SCID mice hosting tissue recombination orthotopic grafts having 22Rv1 prostatic epithelia with human carcinoma associated fibroblasts (CAF).

We expect that our second year studies will allow us to refine the activities of the newly defined small molecule inhibitor described above, while enabling the completion of studies performed over the first year using Siah2 inhibitory peptide (130B3).

2. KEYWORDS

Prostate cancer (PCa); castration resistant PCa; neuroendocrine PCa; Siah1/2; ubiquitin ligases; androgen receptor; HIF1α, CRPC, NEPC, patient derived xenograft (PDX); Shionogi model

3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1: Develop and assess the activity of Siah2 inhibitors
Major Task 1: Further assessment of SBI-601.
Major Task 2: Develop additional derivatives that exhibit superior biophysical properties.
Major Task 3: Assess SBI-601 analogs in benchmark pharmacology, pharmacokinetic, and toxicology studies in cultured cells
Major Task 4: Select best performing SBI-601 analogs (3–5) for studies in mice.
Major Task 5: Select best SBI-601 analog for *in vivo* assessment in PCa mouse models.

Milestone #1: Identify at least one small molecule that is equal if not more potent than SBI-601 for use in Specific Aims 2 and 3. This has been completed on schedule.

Specific Aim 2: Test available Siah2 inhibitors in relevant PCa cultures
Major Task 1: Assess Siah2 inhibitors in relevant NEPC cultures.
Major Task 2: Assess Siah2 inhibitors in relevant ACP-NE cultures.
Major Task 3: Assess Siah2 inhibitors in relevant CRPC cultures.

Milestone #2: Establish efficacy of Siah2 inhibitors in each of the tumor models and identify whether inhibition of Siah2 alone or in combination with currently used drugs has equal or preferable effect on one of the major PCa types assessed.

Specific Aim 3: Test Siah2 inhibitors in PCa models in vivo
Major Task 1: Determine the effect of Siah2 inhibitors (alone and in combination with existing therapies) in xenograft models of castrate resistant, neuroendocrine, and metastatic PCa.
Major Task 2: Test the efficacy of Siah antagonists in the prevention of castration therapy resistance development in novel transgenic and xenograft model systems.
Major Task 3: Determine the effect of Siah2 inhibitors on prostatic and bone metastatic stromal microenvironment on CRPC development.
Major Task 4: Evaluate the efficacy of Siah2 inhibitors on PDX of PCa.
Major Task 5: Determine the ability of Siah2 inhibitor to inhibit CRPC conversion to NE phenotypes when combined with existing therapies.

Milestone #3: We will establish which of the different PCa tumors best responds to Siah2 inhibition, alone or in combination with currently available therapies, monitoring development, progression (metastasis) as well as the conversion of CRPC to NE.

What was accomplished under these goals?

Specific Aim 1 – Develop and assess the activity of Siah2 inhibitors

Major Task 1: Further assessment of SBI-601(Dr. Ronai and Dr. Pinkerton)

This task has been fully completed, as we finalized the characterization of SBI-601, substantiating its effects on prostate cancer cells in culture. These assays included colony forming efficiency, soft agar growth, and assessment of Siah-signature biomarkers, including HIF1α and select AR-target genes. This analysis also raised initial concerns regarding the possibility that there may not be a direct effect of SBI-601 on Siah1/2, which lead us to develop alternative inhibitors for these ubiquitin ligases.

Major Task 2: Develop initial additional derivatives that exhibit superior biophysical properties (Dr. Ronai and Dr. Pinkerton)

The parent and newly developed analogs were subjected to a series of rigorous assessments per their effect on Siah1/2 in both culture and *in vitro*, using purified proteins. Unfortunately, all our efforts

to establish direct interaction or an effect of the parent or analog compounds on Siah2 failed. This led us to conclude that these compounds elicit their biological effect via a different mechanism, which although mimicked Siah1/2's own signature, does not engage Siah1/2 directly. This conclusion, coupled with limited effect in *in vivo* assessments (see below) prompted us to initiate new campaigns for the development of Siah1/2 inhibitors.

Thus, over the subsequent eight-months, we devoted significant efforts towards the two following tasks, which have resulted in promising positive outcomes.

First, over the second year of our studies, we characterized these newly identified small molecules, and repeated the screen in a more refined manner where a full length Siah1 protein was generated and purified for thermal shift assay assessing 80 initial hits and newly added 500 defined inhibitors. Of those, we selected a single inhibitor for further assessment. As shown below, the newly identified inhibitor possesses superior properties over former Siah2 inhibitors identified so far, generating a substantial excitement about their possible utility. The analysis (shown in Figures 1–5) demonstrates the effect of the select compound (#11) on the expression of HIF1α, a surrogate marker for the effect on inhibiting Siah. The ability to inhibit the ubiquitin ligase activity of Siah was also measured, as shown, demonstrating the effective inhibition achieved *in vitro*, using purified proteins, further testimony for the effectiveness of the select small molecule inhibitor. The effect on the proliferation of prostate cancer cells is shown (Figure 3), and on colony formation (Figure 4), demonstrating effectiveness on cell growth with potent toxicity.

Second, we set to advance a Siah inhibitory peptide that we recently developed in parallel, as summarized in our 2013 publication in *Chemical Biology*. At that stage, the inhibitory peptide was 35-45 amino acids long, although the backbone of the actual inhibitory peptide was about 11 amino acids. This peptide was limited to culture work due to its length and the difficulty to produce it in large amounts hindered possible assessment *in vivo*. We thus initiated a campaign to improve on the Siah1/2 inhibitory peptide, which was performed over an eight-month period through a series of six iterative steps. Therein we deleted the penetratine sequence with modified amino acids allowing membrane penetration, by reducing the polyproline linker, without impacting the effect and efficacy of its ability to inhibit Siah, and assuring that the peptide will have a long half life in the cells. These changes were successfully incorporated and at present we have generated a short peptide that effectively inhibits Siah1/2 in culture as well as *in vivo* (130B3). The selectivity and specificity of this peptide to Siah1/2 is secured through the design to force covalent binding of the peptide to Siah1/2 protein. Such covalent association was confirmed in Maldi-TOFF based analysis and more so, in structural studies where the crystal structure of Siah1/2 together with its inhibitory peptide were mapped.

We have thus synthesized a new series of Siah-inhibitory peptides; of the six new peptides, one has demonstrated strongest binding (peptides designed for covalent binding) (Figure 5). The newly selected peptide is currently modified to test for possible improvement and in parallel produced in quantity that will allow its evaluation in animal studies, with the help of Drs. Gleave and Bhowmick. *Figure 5 depicts the effectiveness of the newly synthesized peptide on the Siah in cultured cells, exhibiting strong covalent binding to Siah in vivo.*

Major Task 3: Assess SBI-601 analogs in benchmark pharmacology, pharmacokinetic, and toxicology studies in cultured cells (Dr. Ronai and Dr. Pinkerton)

We expect to extend these studies to perform benchmark pharmacology, pharmacokinetics and toxicology to the most potent small molecule of the newly identified series we currently designate as # 11 Siah inhibitor.

Major Task 4: Select best performing SBI-601 analogs (3–5) for studies in mice (Dr. Ronai and Dr. Pinkerton)

We subjected the SBI-601 and SBI-852 for analysis in mice, as proposed. We found that the effect of these compounds on in vivo growth of the PC tumor CDW22RV1 is limited. This finding, together with the lack of our ability to establish direct effect on Siah ubiquitin ligase activity, prompted the alternative routes we undertook.

Of note, one of the interesting observations made in our in vivo assessment, is the ability of SBI-852 to attenuate the neuroendocrine phenotype seen in RV1 cells in vivo. Further, a similar observation was made by our colleague, Dr. Bhowmick, at Cedars-Sinai (Figure 6, 7).

With the help of Dr. Pinkerton, we expect to design and evaluate a series of new analogues for Siah inhibitor # 11 (Figures 1-5), which will be completed within the current funding period.

Major Task 5: Select best SBI-601 analog for in vivo assessment in PCa mouse models (Dr. Ronai, Dr. Liddington, Dr. Bhowmick and Dr. Gleave)

We performed an initial assessment of the Siah1/2 inhibitory peptide in both culture and *in vivo*, using sc injected RV1 cells and using orthotopically injected cells. We administered the peptide over a three-week period, using daily iv injections (five days injection with two days off before the next series of five daily injection proceed). In both, the orthotopic and sc tumors, we found that the peptide (administered along the same protocol for both modes) effectively inhibited the growth of the prostate tumor *in vivo*. Notably, a substantially greater effect was observed in the orthotopically injected tumor cells, which failed to produce a tumor, compared with the control. In the case of the sc injected tumor, the degree of inhibition was about 50% over a three-week period. In all cases, the peptide was administered iv at a dose of 10 mg/kg. In vivo assessment of the peptide effect on additional PC cultures will be performed over the remaining of the funding.

To produce sufficient material for in vivo studies, we synthesized and purified 1 gram of the Siah1/2 inhibitory peptide, which has now been distributed to both Dr. Bhowmick and Dr. Gleave.

Milestone 1: Identify at least one small molecule that is equal if not more potent than SBI-601/ SBI-852 for use in Specific Aims 2 and 3.

This milestone has been reached as we identified and confirmed the specificity and effectiveness of a Siah1/2 inhibitory peptide, which was successfully modified to enable its effectiveness *in vivo*.

Specific Aim 2 – Test available Siah2 inhibitors in relevant PCa cultures

Major Task 1: Assess Siah2 inhibitors in relevant NE-PC cultures (Dr. Ronai, Dr. Bhowmick and Dr. Gleave)

We have extended a new screen and identified a potent new small molecule, which exhibits superior properties as per inhibition of Siah in vitro and in cultures. This inhibitor will be assessed *in vivo* models in Drs. Bhowmick and Gleave laboratories.

Major Task 2: Assess Siah2 inhibitors in relevant ACP-NE cultures (Dr. Ronai, Dr. Bhowmick and Dr. Gleave)

These assessments are now performed for the newly identified small molecule inhibitor of Siah, with initial promising results.

Major Task 3: Assess Siah2 inhibitors in relevant CRPC cultures (Dr. Ronai, Dr. Bhowmick and Dr. Gleave)

We expect to perform these studies using the newly identified Siah inhibitor during the remaining funding period for this grant.

Milestone 2: Establish efficacy of Siah2 inhibitors in each of the tumor models and identify whether inhibition of Siah2 alone or in combination with currently used drugs has equal or preferable effects on one of the major PCa types assessed.

We expect to perform these studies using the newly identified Siah inhibitor during the remaining funding period for this grant.

Specific Aim 3 – Test Siah2 inhibitors in PCa models in vivo

Major Task 1: Determine the effect of Siah2 inhibitors (alone and in combination with existing therapies) in xenograft models of castrate resistant, neuroendocrine, and metastatic PCa (Dr. Ronai, Dr. Bhowmick and Dr. Gleave)

Further progress has been made using the Siah inhibitory peptide, as reported by Drs. Gleave and Bhowmick. We expect that these studies will be also completed with the newly identified small molecule, as soon its characteristics for in vivo studies have been mapped.

Major Task 2: Test the efficacy of Siah antagonists in the prevention of castration therapy resistance development in novel transgenic and xenograft model systems (Dr. Bhowmick and Dr. Gleave)

Dr. Gleave's lab selects to pre-screen Siah2 protein levels in the Shionogi model. -- 100% fulfilled.

In the last term we analysed mRNA and protein levels of Siah2 and NEPC markers in pre-existing Shionogi tumor tissues before and post castration. Western blotting against HIF1α was not finished at that stage. This term we tested another 2 commercial antibodies against HIF1α but again both of them didn't show specific bands in Shionogi tumor samples. Considering the short half-life of HIF1α protein (only about 5 min under normoxia), it's quite possible that the HIF1α protein may have been degraded in the Shionogi samples during the sample preparation procedure and freeze/thaw cycles. The antibodies we tested include: Cayman (No. 10006421), Cell signaling technology (No. 3716), Abcam (ab19382) and Novus Biologicals (NB100-131). We will not try more antibody for HIF1α western blot in these samples.

Conclusions: SIAH2 protein is acutely induced in Shionogi tumors after castration. Neuroendocrine (NE) markers such as NSE and SYP are also enhanced in the Shionogi tumors post castration. Therefore Shionogi tumor offers a valuable platform to study the effect of inhibiting SIAH2 on the progression of castration resistance and development of neuroendocrine phenotype.

Dr. Gleave's lab selected to investigate if a iah2-inhibiting compound 852 affects repression and recurrence of Shionogi tumor after castration. ----100% fulfilled.

We have conducted the animal work according to the proposal and analysed tumor growth upon the compound 852 treatment. As shown in Figure 8A, we didn't observe significant differences among the vehicle and compound SBI-852 treated groups. Comparison on SIAH2 protein levels among the vehicle and 130B3-treated groups suggested that the compound SBI-852 didn't potently repress

SIAH2 protein levels under the condition tested (up to 40mg/kg, 3 doses/week for about 4 weeks) (Figure 8B).

Conclusions: The compound SBI-852 didn't potently repress SIAH2 under the conditions tested in our *in vivo* model. We will focus on other approaches (for example, SIAH2 inhibiting peptide 130B3) to target SIAH2 in the *in vivo* studies.

Dr. Gleave's lab selected to investigate if a Siah2-inhibiting peptide 130B3 affects repression and recurrence of Shionogi tumor after castration. ---- 75% fulfilled.

The animal work has been performed according to the proposal. Three-week treatments with 10mg/kg of 130B seemed to show a trend of delaying the growth of Shionogi tumor, but the statistical analysis didn't confirm a significant effect (Figure 9A). This is probably due to not enough numbers of animals remained at the very end point. Treatment with 130B3 seemed to slightly repressed SIAH2 protein but had no effect on the protein levels of NE markers NSE, CHGA, and SYP (Figure 9B).
The data described above indicates that 130B3 in Shionogi model looked very promising, however, further effort needs to be conducted to re-tune the experimental conditions to explore if inhibiting SIAH2 with 130B3 may affect the progression of Shionogi tumor growth. Therefore, we proposed a second assay based on the first experiment, included two major adjustments. One is to recruit more animals for each group (15 instead of 10), hoping this will allow us for dedicated statistical analysis. The other one is to start the 130B3 treatment DAILY for the first 5 days (rather than 3 dose/week) and then follow with 3 dose/week until the sacrifice date. This is because SIAH2 protein level was induced already at the 3rd day after the castration, and daily injection during the first week post castration will be able to achieve a better repression of SIAH2 protein. Currently the Shionogi model has been set up and the 130B3 treatment is undergoing.

Dr. Gleave's lab selected to determine if Siah2 level is induced in NEPC samples. --- 100% fulfilled.

Conclusions: SIAH2 protein levels were highly induced in NEPC tumors comparing to prostate adenocarcinoma, as investigated with immunohistochemistry staining in a tissue microarray set from patients' samples and in the PDX xenograft tumors. This suggests that SIAH2 is highly associated with NEPC and the PDX model could be considered as a valuable *in vivo* study model for targeting SIAH2.

Dr. Gleave's lab selected to investigate if 130B3 retards growth of NEPC in LTL352 and LTL331R models. ---- 100% fulfilled.

The patient NEPC tumor tissue LTL352 was recovered and grafted into the NOD-SCID mice for treatments with vehicle or 130B3. However, we have encountered technical obstacles. The i.v. injection is very challenging with the fur tails in these mice. Furthermore, the LTL352 tumor grew slower than the Shionogi tumor, thus required longer treatment before reaching the end point (3 weeks vs 7 weeks). We started to see the treatment effect by the end of three-week treatment (Figure 10A, day 22) but then the tail veins were not injectable anymore and we have to give the mice a break to allow the tail veins to recover for further injections. After finished the whole treatment period we didn't detect significant effect by 130B3 (Figure 10A). Examination of SIAH2 protein levels with western blot suggested that the treatment didn't achieve decent repression on SIAH2 (Figure 10B). Due to the above technique obstacles we didn't start the assay in another PDX LTL331R model.

Conclusions: Due to the technique challenge of long term i.v. injection to the NOD-SCID mice, we are not capable to study the effect of inhibiting SIAH2 on the growth of NEPC tumors with the PDX

LTL352 (and LTL331R) tumor model at this moment. We may make extra effort to try with other drugs targeting SIAH2 that do not require the i.v. injection in future.

In summary we have identified SIAH2 as a castration resistant- and NEPC- associated protein in the Shionogi and NEPC PDX models. Our *in vivo* assays using the SIAH2 inhibiting compound SBI-852 or inhibiting peptide 130B3 in the Shionogi and PDX LTL352 models have pointed out that the treatment in Shionogi with 130B3 is the most promising approach to explore if targeting SIAH2 is able to retard the CRPC and NEPC progression. We will focus on this model for our future studies.

Major Task 3: Determine the effect of Siah2 inhibitors on prostatic and bone metastatic stromal microenvironment on CRPC development (Dr. Bhowmick)

The Cedars-Sinai study site (Dr. Bhowmick) focused on the rigorous evaluation of novel inhibitors of the ubiquitin ligases Siah1/2—a major pathway involved in prostate cancer (PCa) castrate resistance (CRPC). Over the past year, the SBI-852 compound was tested in orthotopic tissue recombination xenograft models of CRPC. We found that SBI-852 had little effect on the gross tumor volume, but seemed to definitively reduce neuroendocrine differentiation of the tumors generated. This encouraging finding led us to examine the next generation Siah antagonist, 133B3, in a model in conjunction with androgen deprivation therapy. Our past collaborative work has demonstrated that Siah binds and initiates programed degradation of an inactivated form of androgen receptor. Further we identified that Siah is important in the differentiation of prostate cancer to a termed neuroendocrine state, in which it is refractive to conventional castration-based therapy. Thus, blocking Siah activity has the added advantage of preventing castrate resistance—especially important if such a therapeutic is used in combination with currently used androgen ablation methods. Two main experiments performed with 133B3 in the past year included the use of enzalutamide and castration as means of androgen deprivation. In the first study, CRW22Rv1 epithelia were xenografted with human prostatic carcinoma associated fibroblasts (CAF) with in the anterior prostate lobes. The tumors were allowed to progress untreated in the mice until they reached approximately 1 cm^3, by MRI. Then the mice were either treated with enzalutamide alone or treated in combination with 133B3 (10 mg/kg). Figure 6 illustrates the dramatic reduction in tumor size induced by the combination treatment of enzalutamide and 133B3. Interestingly, we found that there was no statistical difference in the fraction of proliferative index of the cells (localized by Ki67) in the tumors of the two treatment groups. However, the TUNEL-positive cells, suggestive of cell death, was significantly greater the combination treatment arm. We found that neuroendocrine differentiation of the tumor induced by enzalutamide, was largely downregulated when 133B3 was administered with enzalutamide (Figure 6). Unfortunately, we lost three mice to toxicity in the 3-week treatment period in this experiment. So in the next experiment, we chose to treat the host mice harboring identical orthotopic CWR22Rv1/CAF tissue recombinant grafts, with a lower dose of 133B3 (5 mg/kg). As before, we allowed the tumors to expand to approximately 1 cm^3 prior to 133B3 treatment. However, instead of giving enzalutamide in this study we castrated the mice as the strategy for androgen deprivation. In this study, one mouse was lost to the treatment. After 3 weeks of treatment, we found the tumors in the castrated mice were grossly similar to those castrated mice given the lower dose of 133B3 (Figure 7). However, the histologic interrogation of the tissues provided encouraging results, similar to that when we used 10 mg/kg 133B3. There was little difference in the treatment groups in the mitotic index (phosphorylated-histone H3). But, neuroendocrine differentiation was downregulated by the 133B3. Conversely, the apoptotic index was significantly greater in the castrated mice given 133B3, compared to castration alone.

In the timeframe of the no-cost extension the Bhowmick laboratory will be testing the third generation Siah inhibitor recently identified by the Ronai laboratory. Further, they will be testing the role of 133B3 and third generation Siah inhibitor on the protein stability of full length and splice variants of androgen receptor (AR) in both the cancer epithelia and its associated stromal fibroblasts. In the last funding

cycle, the critical importance of the role of AR variant expression in the stromal fibroblasts on tumor expansion and castrate resistance was revealed. While we have previously reported that Siah2 is largely expressed in the prostatic epithelial compartment, Siah1 (also targeted by 133B3 and the 3rd generation antagonist) is expressed at some level in the (CAF).

Major Task 4: Evaluate the efficacy of Siah2 inhibitors on PDX of PCa (Dr. Bhowmick and Dr. Gleave)

We expect to perform these studies using the newly identified Siah inhibitor during the remaining funding period for this grant.

Major Task 5: Determine the ability of Siah2 inhibitor to inhibit CRPC conversion to NE phenotypes when combined with existing therapies (Dr. Ronai and Dr. Gleave)

We expect to complete this task over the remaining period of funding.

Milestone 3: We will establish which of the different PCa tumors best responds to Siah2 inhibition, alone or in combination with currently available therapies, monitoring development, progression (metastasis) as well as the conversion of CRPC to NE.

We expect to perform these studies using the newly identified Siah inhibitor during the remaining funding period for this grant.

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

Nothing to report.

How were the results disseminated to the communities of interest?

The results of the studies performed during the first year of this funding period were discussed in professional meetings including the ubiquitin conference that was held in Croatia in September 2015. A discussion with the prestigious forum at UCSD, named Oher, provided an opportunity to discuss our approach and results with the greater community members (May 2015).

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

We made changes during the second year of funding to ensure we can achieve the goals of this proposal. We implemented two parallel approaches, thereby securing our ability to complete these goals, as outlined above. We expect that our work will enable to successfully complete the originally planned experiments. The significant progress in identifying new small molecule inhibitors for Siah (#11) will be now assessed, as part of this grant coverage, in *in vivo* models by the Bhowmick and Gleave laboratories.

4. IMPACT

The work performed during the second year of this project can be considered as a paradigm shift in cancer research in general and prostate cancer in particular. To this day there is no specific inhibitor of a ubiquitin ligase that can be administered *in vivo*, as we have now accomplished by the work we performed with the Siah1/2 peptide inhibitors. We have further used a state-of-the-art approach to screen for novel Siah1/2 small molecule inhibitors, which led to identification of several promising compounds we currently subject to rigorous assessment as part of the originally planned studies.

The collaboration between the three labs (Ronai, Gleave, and Bhowmick) will be of particular importance during the second year of this proposal when they are evaluating the significance of inhibiting Siah1/2 for the most aggressive prostate cancer, CRPC and NE type. CRPC and NEPC remain major obstacles in treatment of advanced PCa. Our study will explore roles of Siah2 on CRPC development and on growth of established NEPC. The PDX and Shionogi models recruited in our study present highly clinical relevance and provide unique systems on evaluating the efficacy of agents targeting progress of advanced disease. Data obtained from our study using Siah2 inhibiting agents, compound 852 and peptide 130B3, will help to determine if targeting Siah2 may bring significant benefit to patients with CRPC and NEPC.

A year later, the community still lacks a potent inhibitor for ANY ubiquitin ligase. Our work faced technical challenges, which we have encountered at the level of potency, especially in moving our assessments from culture to *in vivo* studies. We have thus re-ignited the efforts to identified potent inhibitors for the Siah ubiquitin ligases, which enabled the identification of new small molecule inhibitor with promising properties, we have not seen before. Work is expected to be completed by way of *in vivo* assessment, and is expected to provide invaluable resource for development of inhibitors to ubiquitin ligases, as well as innovative approach for inhibition of Siah ubiquitin ligases *in vivo*.

What was the impact on the development of the principal disciplines of the project?

The need to alter our original plan due to disappointing results forced the incorporation of two alternate approaches, each is unique and first in class on its won, which were successfully implemented within this short time allowing progress of the originally planned studies.

What was the impact to other disciplines?

Our work over the first year have secured our ability to perform our planned studies in the best possible way, using distinct novel approaches which offer a paradigm shift in development and therapeutic modalities for PC.

What was the impact on technology transfer?

We expect that the outcome of our work during the first year will offer novel intellectual properties and technologies that will be disseminated to the greater community.

What was the impact on society beyond science and technology?

The ability to develop a first in class reagents to inhibit PC

5. CHANGES/PROBLEMS

Changes in approach and reasons for change.

As outlined above, we had to alter the original plan to develop the SBI-601 and its analogs further given their poor performance *in vivo* and in light of the inability to confirm their direct impact on the Siah1/2 ligases. This has prompted our development of two alternate approaches that appear to be successful and secure the continued work, as originally planned, in defining a new class of inhibitors to the most aggressive forms of PC.

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

Nothing to report.

Changes that have a significant impact on expenditures.

The change in the approach described above has impacted the expenditures of this project, which was reflected in cost to synthesize sizeable amount of Siah1 protein, the cost of synthesizing number of Siah1/2 inhibitory peptides, the cost of synthesizing large amount of the select Siah1/2 inhibitory peptide (1 gram) and the cost of establishing and performing the thermal shift assay on 32,000 compounds.

As noted, during the second year of funding we have advanced these studies by performing a secondary screen for full-length Siah protein modulators, enabling the selection of single hit for extensive characterization – from development and assessment of analogues to *in vitro*, culture and *in vivo* analyses.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to report.

6. PRODUCTS

Publications, conference papers, and presentations

The results of the work performed over the first year has so far been described in a couple of ubiquitin and cancer meetings, including the EMBO ubiquitin conference in Croatia in September 2015, and a ubiquitin workshop in China in June this year. Patent applications are expected upon further refining the Siah1/2 inhibitory peptide and the small molecules identified in the course of the HTP screen we performed this year.

Journal Publications

Nothing to report.

Books or other non-periodical, one-time publications

Nothing to report.

Other publications, conference papers, and presentations

Nothing to report.

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.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Ronai Lab

Name:	Ze'ev Ronai
Project Role:	Principal Investigator
Researcher Identifier:	
Nearest person month worked:	1
Contributions to Project:	PI
Funding Support:	N/A

Name:	Yongmei Feng
Project Role:	Staff Scientist
Researcher Identifier:	
Nearest person month worked:	2
Contributions to Project:	Performed assessment of inhibitors in culture and *in vivo.*
Funding Support:	N/A

Gleave Lab

Name:	Fan Zhang
Project Role:	Research Associate
Researcher Identifier:	
Nearest person month worked:	2
Contributions to Project:	Dr. Zhang is the project manager for this study. Dr. Zhang has been communicating with Ronai's lab, Gleave's lab and Wang's lab for the protocol preparation. Dr. Zhang has been manipulating the animal work together with the animal staff at Vancouver Prostate Centre, and Dr. Zhang is in charge of the data analysis and report preparation.
Funding Support:	N/A

Name:	Alexander Kretschmer
Project Role:	Post-doctoral Researcher
Researcher Identifier:	
Nearest person month worked:	2
Contributions to Project:	Dr. Kretschmer has been taking the duty to monitor tumor growth and animal body weight and helps to harvest tissues at the end point of treatments. Dr. Kretschmer is also heavily involved with data analysis.
Funding Support:	N/A

Name:	Mary Bowen
Project Role:	Research Assistance on animal work
Researcher Identifier:	
Nearest person month worked:	2
Contributions to Project:	Ms. Bowen sets up the animal models and performs the drug treatments.
Funding Support:	N/A

Name:	Dong Lin
Project Role:	Research Associate in Dr. YZ Wang's lab
Researcher Identifier:	
Nearest person month worked:	1
Contributions to Project:	Dr. Lin has been cooperating to set up the PDX LTL352 and 331R tumor models for the study.
Funding Support:	N/A

Name:	Darrell Trendall
Project Role:	Research Associate on animal work
Researcher Identifier:	
Nearest person month worked:	2
Contributions to Project:	Mr. Trenall performs the drug administration.
Funding Support:	DoD 0.25 FTE

Name:	Brian Li
Project Role:	Student of Gleave Lab
Researcher Identifier:	
Nearest person month worked:	2
Contributions to Project:	Mr. Li has been helping Dr. Kretschmer for animal monitoring and Dr. Zhang for animal sample analysis (western blot, …)
Funding Support:	DoD 0.25 FTE

Name:	Ladan Fazli
Project Role:	Research Scientist-Pathologist
Researcher Identifier:	
Nearest person month worked:	1
Contributions to Project:	Dr. Ladan Fazli helps for the tissue microarray preparation and IHC staining and scoring
Funding Support:	D0D 0.5 FTE

Bhowmick Lab

Name:	Manisha Tripathi
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person month worked:	8.4
Contributions to Project:	Performed the surgeries and helped in the analysis of the tissues.
Funding Support:	N/A

Name:	Rajeev Mishra
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person month worked:	3
Contributions to Project:	Helped in the surgical procedures, treatment of the mice, and analysis.
Funding Support:	N/A

Name:	Neil Bhowmick
Project Role:	Principal Investigator
Researcher Identifier:	
Nearest person month worked:	0.5
Contributions to Project:	Design and analysis of the data. Share responsibility of overall running of the project and coordinating with Drs. Gleave and Ronai.
Funding Support:	N/A

As we have developed new peptides and established the ability to perform novel screen for Siah1/2 inhibitors we have engaged additional in-house collaborators that enabled us to secure our progress to date. This includes Dr. Christian Hassig (working with Dr. Anthony Pinkerton) to oversee the development of the HTP screen and its performance, as well as Drs. Surya De and Maurizio Pellecchia, who assisted in design and synthesis of the novel Siah1/2 inhibitory peptides.

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

Ze'ev Ronai, Initiating PI

Grants that have ended:

1 R01 CA188372-01A1 (PI: Ronai, Z.) 07/01/15–05/31/20 1.2 calendar (10.0%)
NIH/NCI
Understanding and Targeting the Glutamine Carrier SLC1A5 in Breast Cancer
Goals: The major goal of this project is to advance the understanding of Gln metabolism in BCa and provide the foundation for novel stratification methods and therapeutic modalities for BCa.
Specific Aims: (1) Identify RNF5-dependent and -independent transcriptional, translational, and post-translational events regulating SLC1A5/38A2 availability and activity in representative BCa cultures (2) Establish the biological significance of SLC1A5/38A2 expression in BCa cells for cellular metabolism, mitochondrial dynamics and function, autophagy, growth, and response to therapy (3) Using BCa tumor samples, circulating tumor cells and TMAs we will determine the relationship between BCa expression of SLC1A5/38A2 and RNF5, the response to treatment, and disease outcome.
No overlap
Grants Specialist: Sarah Lee
National Institutes of Health
900 Rockville Pike
Bethesda, MD 20892
Phone: (240) 276-6280

E-mail: sarah.lee@nih.gov

1 R01 CA179170-02 (PI: Ronai, Z.) 09/30/13–07/31/16 1.2 calendar (10.0%)
NIH/NCI
PDK1 as a Novel Target in Melanoma
Goals: The major goal of this project is to determine whether PDK1 is an important signaling node required for melanoma development and progression.
Specific Aims: (1) Determine the role of PDK1 in Nras and Braf mutant melanomas harboring wild-type Pten (2) Characterize the molecular mechanisms underlying PDK1 control of melanoma development and progression (3) Identify biomarkers of PDK1-sensitive tumors and determine the clinical significance of PDK1 expression in melanoma.
No overlap
Grants Specialist: Cammie La
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (240) 276-6323
E-mail: cl311z@nih.gov

1 R01 CA172017-02 (PI: Ronai, Z.) 07/01/13–04/30/16 1.2 calendar (10.0%)
NIH/NCI
ATF2 Oncogenic Addiction in Melanoma
Goals: The major goal of this project is to identify unrecognized pathways for melanoma development that could be exploited for the development of innovative therapeutic modalities.
Specific Aims: (1) Determine the mechanisms and significance of ATF2 addiction to PKC epsilon in melanoma (2) Evaluate the oncogenic and tumor suppressor functions of ATF2 using conditional ATF2 knock-in mice (3) Identify natural compounds that promote nuclear export of ATF2 in melanoma cells.
No overlap
Grants Specialist: Barbara A. Fisher
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (301) 631-3012
E-mail: bfisher@mail.nih.gov

W81XWH-14-1-027 (PI: Ronai, Z.) 09/30/14–09/29/16 0.6 calendar (5.0%)
DoD
Siah1/2 Ubiquitin Ligases in ER Stress Signaling in Melanoma
Goals: The major goal of this project is to test the efficacy of Siah2-inhibiting compounds in melanoma cells and in relevant genetic and xenograft models.
Specific Aims: (1) Establish the significance of the Siah2-hypoxia-ER stress regulatory axis in melanoma development and response to chemotherapy of a subset of melanoma (2) Determine the effect of inhibitors of the Siah2-ER stress axis on melanoma development and response to chemotherapy.
No overlap
Grants Officer: Patricia Roth
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702
Phone: (301) 619-7452
E-mail: patricia.a.roth.ctr@mail.mil

Pending grants that are now active:

1 R35 CA197465-01 (PI: Ronai, Z.) 07/01/15–06/30/22 6.0 calendar (50%)
NIH/NCI
Rewired Signaling at the Nexus Melanoma Metastasis and Resistance
Goals: The major goal of this project is to establish novel mechanisms underlying tumor plasticity, enabling the development of novel agents for predicting, monitoring, and preventing tumor metastasis and resistance.
Specific Aims: (1) to continue to investigate how PDK1 and its downstream targets contribute to melanoma metastasis and drug resistance. (2) Develop and evaluate first-in-class Siah inhibitors for prostate cancer and melanoma metastasis and resistance
No overlap
Scientific Review Officer: Michael B. Small
National Institute of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (240) 276-6438
E-mail: smallm@mail.nih.gov

2 P01 CA128814-06A1 (PI: Ronai, Z.) 04/01/16–03/30/21 1.56 calendar (13%)
NIH/NCI
ER Stress and Mitochondrial Biogenesis in Melanoma
Goals: The major goal of this project is to develop new paradigms enabling a better understanding of melanoma plasticity—the underpinning of its reprogramming—and offer innovative therapeutic approaches to treat invasive and drug resistant disease.
Specific Aims: (1) Define the role of UPR components in melanoma metastasis and resistance to therapy. (2) Determine the role of proliferator-activated receptor y coactivators and downstream signaling in melanoma and acquisition of drug resistance (3) Elucidate the role of regulation of a feedback loop between MITF and ATF4, and the resulting downstream metabolic alterations, in acquisition of the invasive and drug-resistant phenotype of melanoma.
No overlap.
Scientific Review Officer: Caterina Bianco
National Institute of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (240) 276-6459
E-mail: biancoc@mail.nih.gov

1 R01 CA202021-01A1 (PI: Ronai, Z.) 07/01/16–06/30/21 0.65 calendar (5.4%)
NIH/NCI
Control of Protein Synthesis by the UPS Under Stress
Goals: The major goal of this project is to establish the importance and significance of select UPS components in a novel regulatory network that controls protein synthesis during cellular stress, and establish its role in BCa using a combination of BCa cultures and xenografts, RPPA technology, and TCGA dataset mining.
Specific Aims: (1) Establish the physiological significance of the RACK1-JNK-eEF1A2 regulatory axis to the cellular stress response, growth and therapeutic response of BCa (2) Assess the role of stress-induced polysomal recruitment of Nedd8-Culllin machinery in regulating the decay of newly synthesized proteins in BCa (3) Determine the importance of UBQLN1 recruitment to polysomes in regulating newly synthesized proteins under stress conditions and in modulating the response of BCa to therapy.
No overlap.

Scientific Review Officer: Nywana Sizemore
National Institute of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (301) 408-9916
E-mail: sizemore@csr.nih.gov

Martin Gleave, Partnering PI (Vancouver Prostate Centre)

Nothing to report.

Neil Bhowmick, Partnering PI (Cedars-Sinai Medical Center)

Grants that have ended:

n/a Knudsen/Freeman (PI; Role: Co-I) 10/01/12–09/30/15 0.12 calendar (1%)
Spielberg Family Cancer Foundation
The Ecosystem of Lethal Prostate Cancer
Goals: The study involves the analysis of DNA methylation status of prostate cancer patient stromal cells in order to better characterize the cancer associated changes leading to lethal prostate cancer progression.
Specific Aims: (1) To identify genomic and transcriptomic signatures of adverse outcome of Gleason grade 4 (GG4) prostate cancer (2) To test whether modified gold nano-rods can be used to quantify methionine metabolites in serum as a means of reporting adverse outcome in prostate cancer (3) To test whether genomic and palmitoyl-proteomic profiles of a primary tumor can be measured in affinity-captured microvesicles (4) To identify a signature of adverse outcome in circulating tumor cells (CTCs).
No overlap.

Pending grants that are now active:

P01 CA098912-11 (Chung; Project 3 Leader) 03/03/2015-02/29/2020 1.2 Calendar (10%)
NIH/NCI
Prostate Cancer Bone Metastasis Biology and Targeting
Goals: To understand the basic biology of lethal prostate cancer progression to bone metastasis, and the design of new targeted therapies such manifestations. Project 3 will delineate stromal- epithelial interaction by characterizing the roles of prostate stroma, and specifically the Notch pathway, in driving PC progression by promoting the emergence of tumor cells expressing a megakaryocyte and osteomimicry phenotype.
Specific Aims: (1) To test whether CTCs and DTCs are the depots for MIC recruitment and reprogramming and contribute to bone and soft tissue metastasis. (2) To determine the molecular features of MICs and bystander cells required for their participation in the recruitment, initiation and reprogramming for distant metastasis. (3) To discover new therapeutic targets in the tumor microenvironment and test small molecule inhibitors and antibodies targeting relevant TFs and signaling pathways.
No overlap.
Contracting/Grants Officer: Robert Maydwell
Grants Management Specialist
National Institutes of Health, FTC-2 West
HHS-NIH-NCI-OGA/Branch B
9000 Rockville Pike, Bethesda, MD 20892
(240)-276-6478

Robert.Maydwell@nih.gov

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

This grant is a joint proposal with the following log numbers and respective award numbers. As such, we will be submitting duplicative reports.

CDMRP Log Number	Grant Agreement Number	Recipient	Principal Investigator
	W81XWH-14-1-0551	Sanford-Burnham Medical Research Institute	Ze'ev Ronai
PC130699P1	W81XWH-14-1-0552	Cedars-Sinai Medical Center	Neil Bhowmick
PC130699P2	W81XWH-14-1-0553	University of British Columbia	Martin Gleave

9. APPENDIX

Figures from Dr. Ronai's lab are shown in Figures 1–5. Figures depicting the studies in Dr. Bhowmick's lab are shown in Figures 6–7. Data from Dr. Gleave's lab are shown in Figures 8–10.

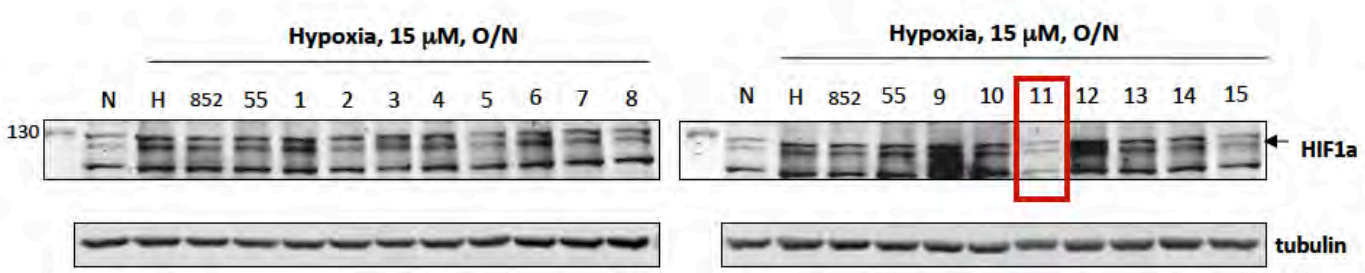
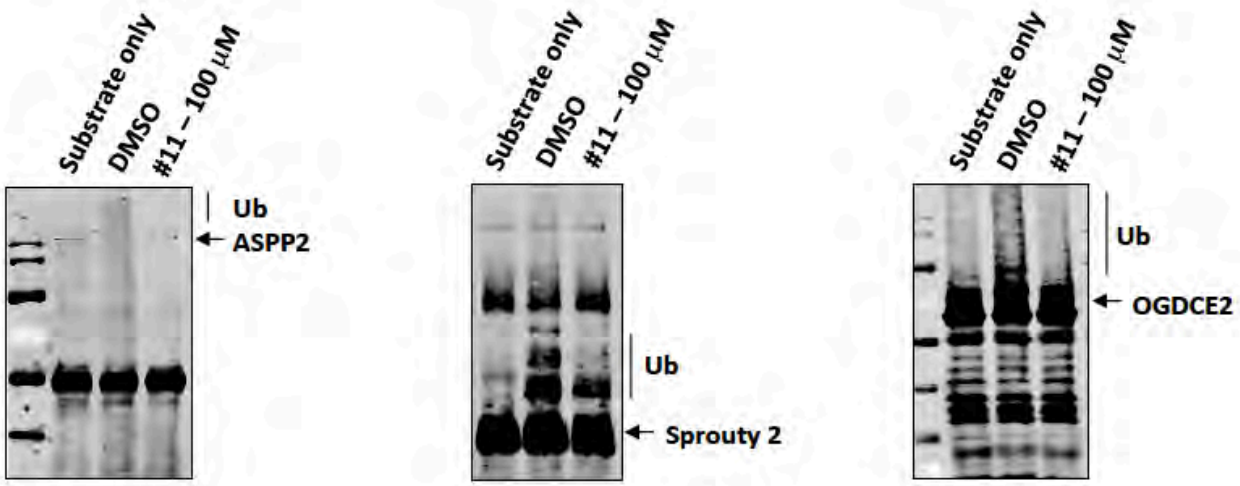


Figure 1. Effect of compound # 11 compared with other Siah inhibitors identified in earlier screening campaigns on HIF1α. Different compounds (15 µM) were added to tumor cells and cultured in 1% oxygen incubator overnight. HIF levels were examined by Western Blot analysis.



* **ASPP2, Sprouty 2 and OGDCE2 are three Siah substrates used in in-vitro Siah ubiquitition assay**

Figure 2. Inhibition of *in vitro* ubiquitination of three Siah substrates. Compound #11 was incubated with different Siah substrates for half an hour followed by the addition of ubiquitination reagents (E1, E2, Ub). The mixtures were then incubated at 37°C for 45 min followed by Western Blot analysis.

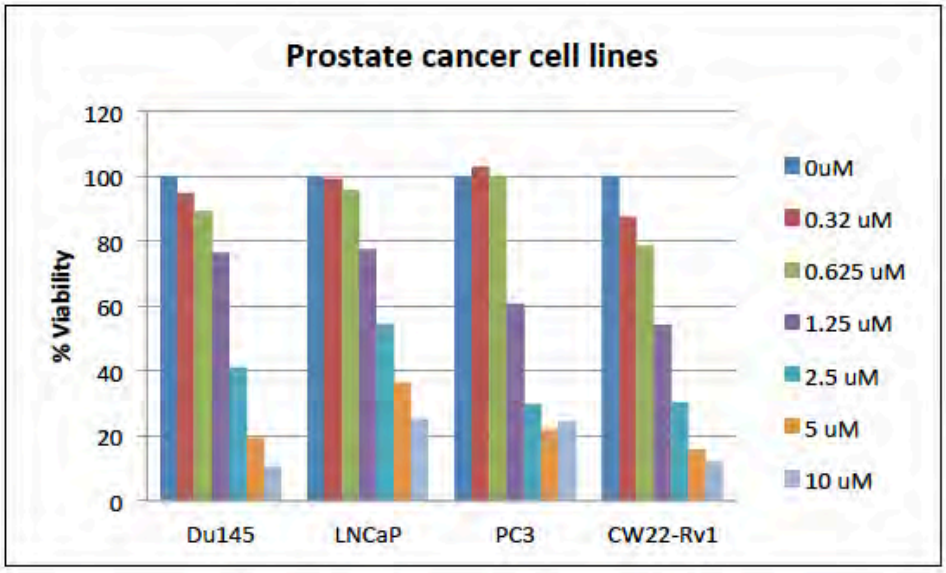


Figure 3. Dose dependent inhibition of prostate cancer cell lines following treatment with compound #11. Compound #11, at the indicated concentrations, was added to different prostate cancer cell lines and viability was assessed 72 h after treatment**.**

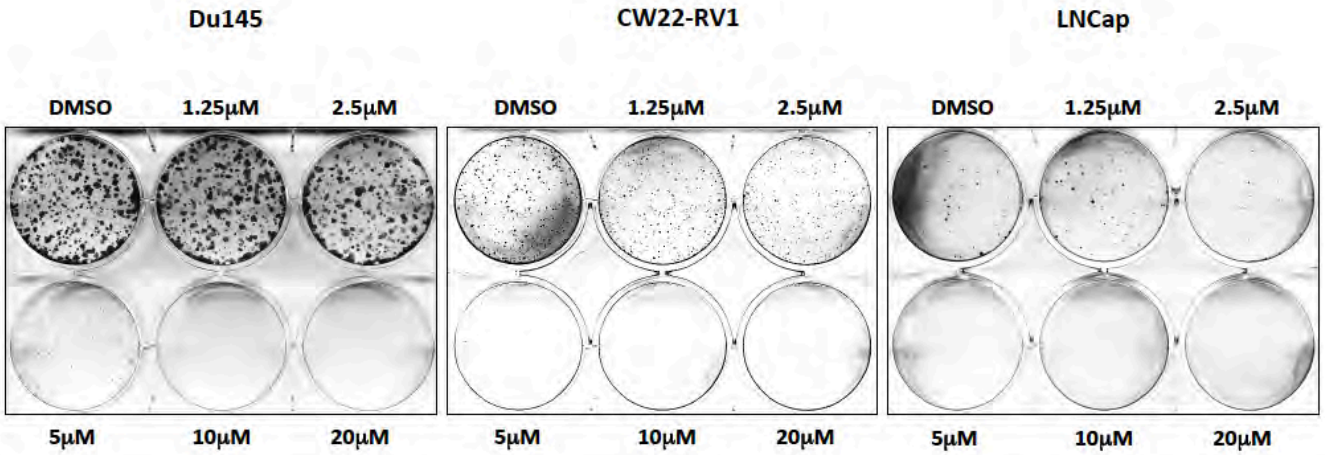


Figure 4. Compound #11 elicits a dose dependent inhibition of colony formation by prostate cancer cells. Compound #11, at the indicated concentrations, was added indicated prostate cancer cell lines. CFE was assessed after 10–14 days.

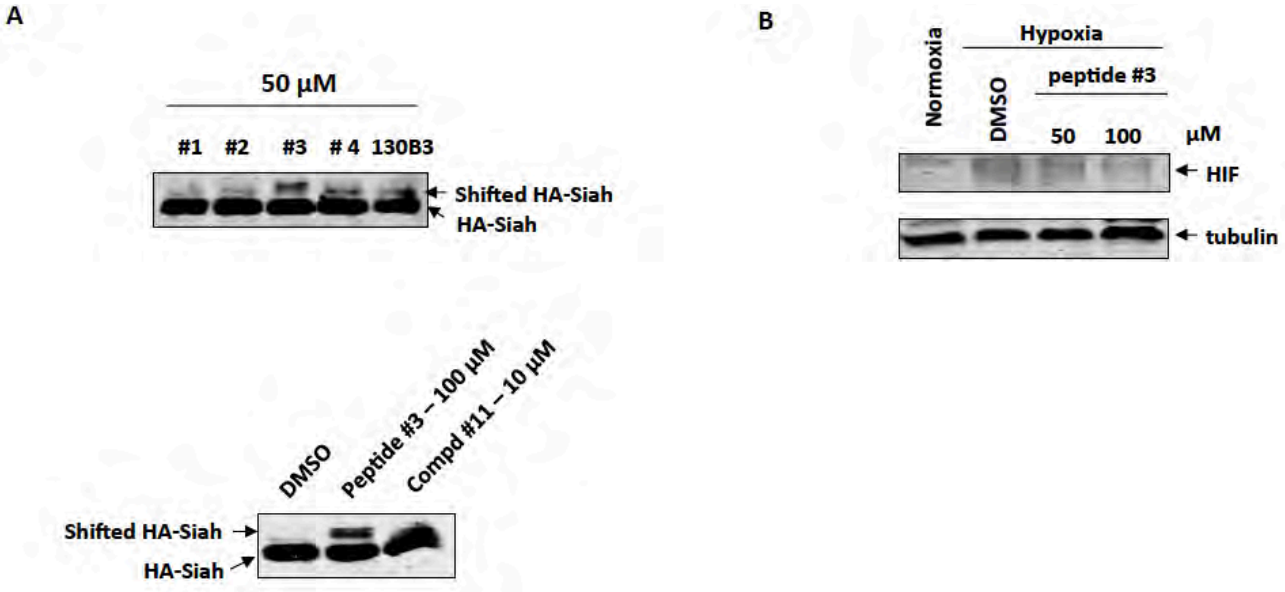


Figure 5. Siah peptide inhibitor #3 could penetrate cells, bind to Siah and inhibit HIF1α induction underhypoxia. (A) Four Siah peptide inhibitors (50 μM) were added to cancer cells (transfected with HA-Siah) and cultured overnight. Cell lysates were subjected to Western Blot analysis. (B) Different concentration of Siah peptide inhibitor was added to cells and cultured under 1% oxygen overnight. Cell lysates were subjected to Western Blot analysis. Data shows efficient conjugation (covalent binding) of peptide #3 to Siah2 in vivo, which is seen even after SDS-PAGE separation compared with previously used peptide 130B3.

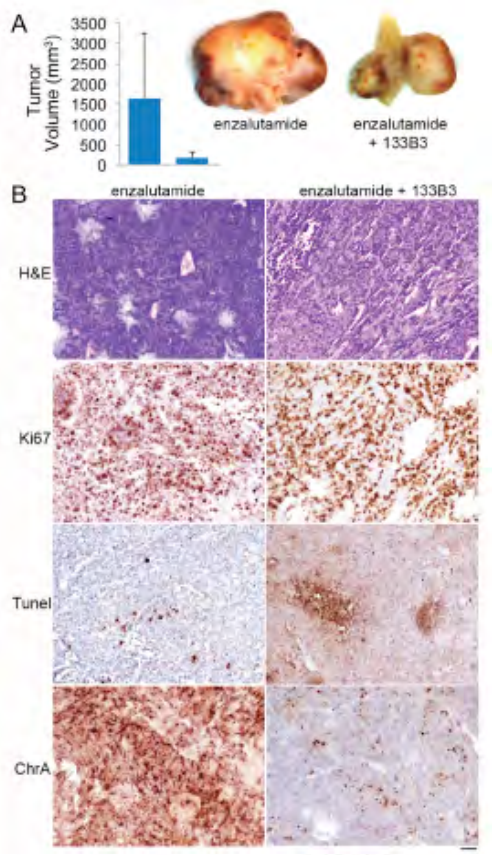


Figure 6. Host mice were treated with enzalutamide alone or enzalutamide and 133B3 (10 mg/kg). The gross tumor size and histologic differences are highlighted.

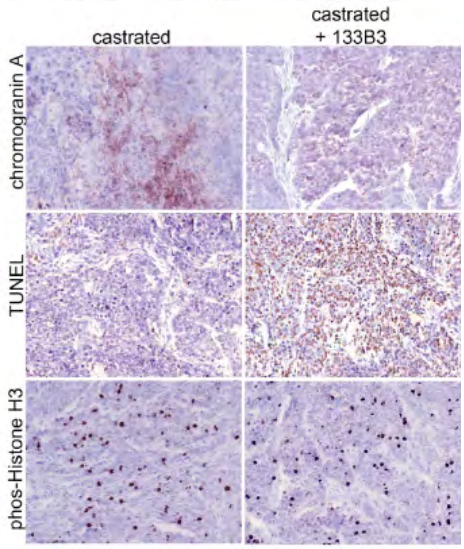


Figure 7. Host mice were just castrated or castrated and treated wit 133B3 (10 mg/kg). The gross tumor size and histologic differences ar highlighted.

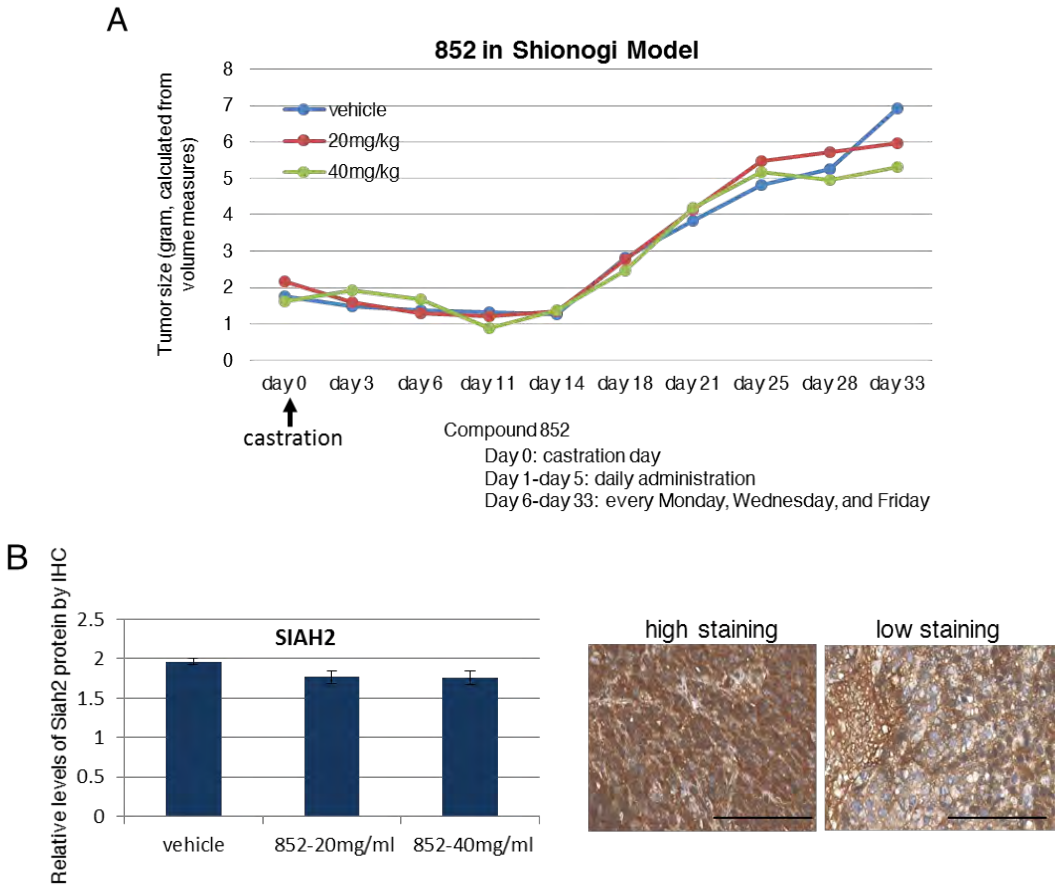


Figure. 8. Effects of Siah2 inhibitor compound 852 on Shionogi tumor progression after castration. 5 x 10^6 of TD-2 cells were injected subcutaneously into DD/S nude mice. When tumors reached 500 mm^3 (2 to 3 weeks after injection) mice were castrated under anesthesia and randomly entered groups for vehicle, 20 mg/kg of compound 852 and 40 mg/kg of compound 852. Treatments were started the day after castration with i.p. injection, daily administration for the first 5 days and then three doses/week for the rest period. (A) Tumor growth was continuously measured until the sacrifice date. (B) SIAH2 protein levels were investigated with immunohistochemistry (IHC) staining on the tumor tissue sections ($p > 0.05$). Left panel: scoring result; right panel, representative images of SIH2 IHC, scale bar: 100 µm.

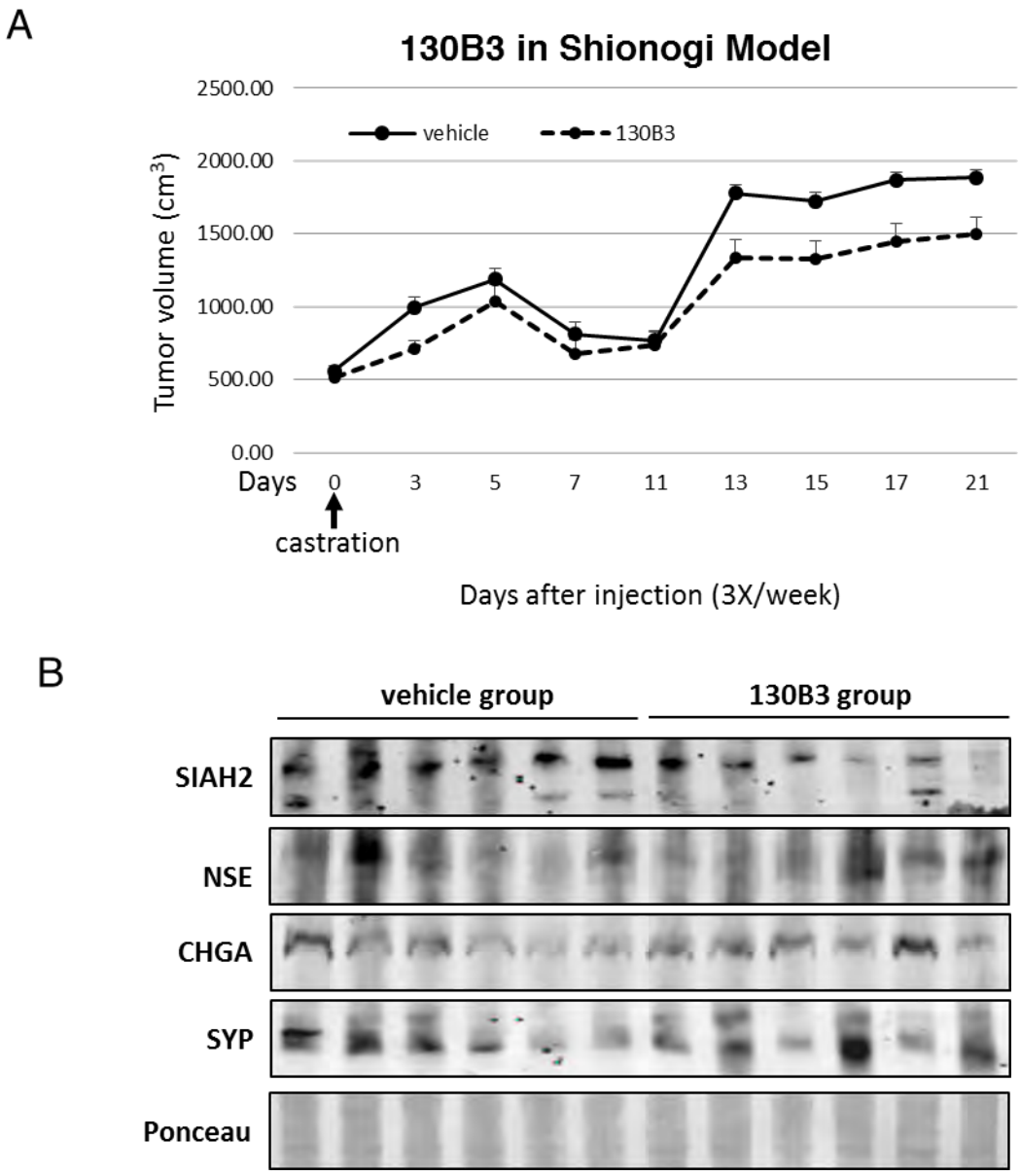


Figure. 9. Effects of Siah2 inhibiting peptide 130B3 on Shionogi tumor progression after castration. 5 x 10^6 of TD-2 cells were injected subcutaneously into DD/S nude mice. When tumors reached 500 mm^3 (2 to 3 weeks after injection) mice are castrated under anesthesia and randomly entered groups for vehicle and 10 mg/kg of 130B3. Treatments were started the day after castration with i.v. injection for 3 doses/week. (A) Tumor growth was continuously measured until the sacrifice date. (B) Protein levels pf SIAH2 and NE markers CHGA, SYP and NSE were investigated with Western Blot in the tumor tissue protein lysates.

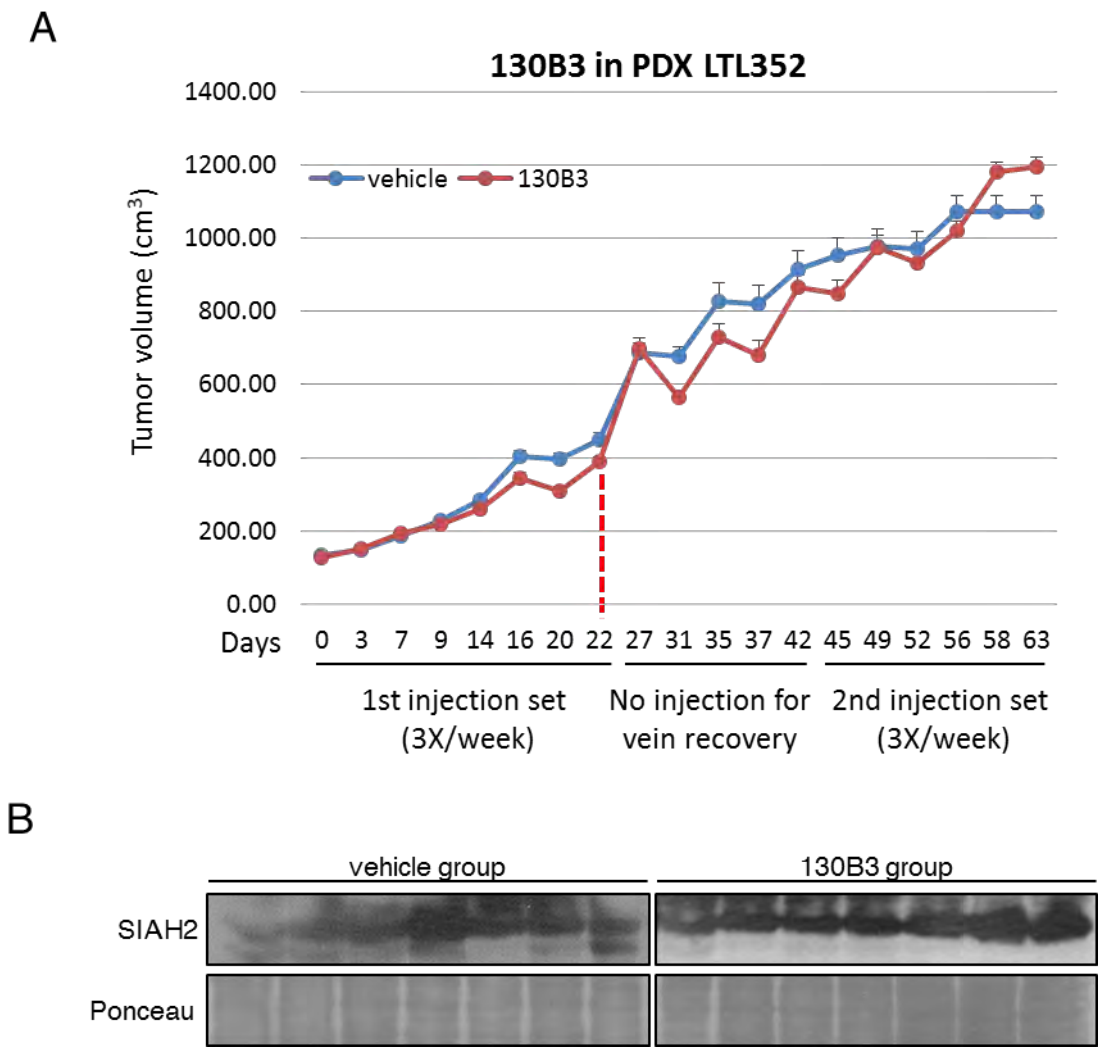


Figure. 10. Effects of Siah2 inhibiting peptide 130B3 in PDX model LTL352. Patient NEPC tumor tissue LTL352 was recovered from liquid nitrogen and maintained in NOD-SCID mice under the renal capsules with supplement of testosterone. Recovered tissue pieces were then grafted under the renal capsules of pre-castrated male NOD-SCID mice. When tumors reached 200 mm^3, mice were randomly grouped for i.v. treatments with vehicle or 10 mg/kg of 130B3. (A) Tumor growth was continuously measured until the sacrifice date. (B) Protein levels of SIAH2 were examined with Western Blot in tumor tissue protein lysates.