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**14. ABSTRACT**

The goal of this project was to develop a novel means to inhibit prostate cancer development and progression. The development of Siah1/2 inhibitors to the ubiquitin ligase Siah1/2 was 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 reagents was performed *in vitro*, in cultures and in genetic models of mouse as in human PDX tumors in our lab as by the Partnering PIs, Drs. Martin Gleave and Neil Bhowmick at the two respective sites. Most of my lab's attention was given to the development and assessment of small molecule inhibitors to Siah2 as a mean to complement the work performed with the inhibitory peptide. The goal has been to secure the first-in-class inhibitor of the ubiquitin ligase that can be used *in vivo* for the inhibition of prostate cancer.

**15. SUBJECT TERMS**

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

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## 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 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 was 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 made significant progress on both fronts, as noted below.

We completed two new high-throughput screens for Siah inhibitors. The thermal shift assay was used to screen for Siah bound small molecules that alter its melting temperature. Out of 32,000 small molecules, we identified 15 for further assessment, including their effect on Siah ubiquitination and the stability of Siah substrates, including HIF1α and ERK phosphorylation (which are regulated by Siah control of PHD1/3 and Sprouty2, respectively). Those selected failed to elicit effective changes in cultured cells and were not pursued for further assessment. Instead, we went back and screened full length Siah that was generated in bacculovirus. This led to performing a screen that focused on the top 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 was found to effectively attenuate the stability of HIF1α, as expected from a Siah inhibitory protein.

In all, our extensive efforts have advanced the understanding of possible small molecule inhibitors that can elicit effective inhibition of Siah *in vitro* and in culture. The greatest obstacle deemed to be at the *in vivo* studies, where effective compounds failed to elicit strong biological activity. The example of SBI-852 is the first, where most promising culture based data did not lead to equally potent data *in vivo*, but

rather disappointment, both in our models, and in the models tested by our collaborators, Drs. Gleave and Bhowmick. Our most promising small molecule inhibitor from all the work performed under the support of this funding is Adapalene and a couple of its derivatives, which appeared to elicit effective inhibition *in vitro*, *in vivo* and to some degree also in mouse models. Here the concern, which needs to be further addressed, is that the active moiety on this compound resembles some of retinoic acid properties, which require further assessment and possible modifications. Yet, the big gap between the culture findings in cell culture and *in vivo* setting led us to also explore an intermediate solution, such as packaging the inhibitors in nanoliposomes, an aspect that is ongoing in our laboratory.

Our biggest take home message, is that the approach we undertook to map possible inhibitors to Siah ubiquitin ligases need to be reconsidered, given the limit efficacy in using small molecules to inhibit protein–protein interactions of the magnitude presented by the Siah ubiquitin ligase complex. This is consistent with work performed by other groups, which aimed at inhibiting Siah and other ubiquitin ligases. Considerations for other approaches, including Celebron-based are being made.

## 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 was fully completed and reported on in previous years.

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

Over the final year of funding, we devoted significant efforts to (i) the further characterization of the above-mentioned inhibitor and (ii) an independent screening campaign to identify new potential inhibitors for Siah ubiquitin ligases.

Our results with the single compound identified in year 2 were extended towards further characterization of the inhibitor (designated as # 11; by structure identified as Adapalene), and in addition, the design and testing of analogues that may be superior to #11. Eight derivatives were identified and tested for the key parameters we have been using in assessing Siah2 inhibitors. Of the different hits, #11 and two of the analogues exhibit potent inhibition of Siah2 – measured by levels of HIF1α expression and by their ability to effectively kill prostate cancer and melanoma cells in culture. These were by far the most potent compounds we have identified to date for Siah inhibition. Not only that the effect was seen on HIF1α expression and viability of melanoma and prostate cancer cells in culture, but these inhibitors also inhibited Siah2 ubiquitin ligase activity *in vitro* (Fig. 1). Further assessment of these compounds was performed *in vitro* and *in vivo*, as outlined below.

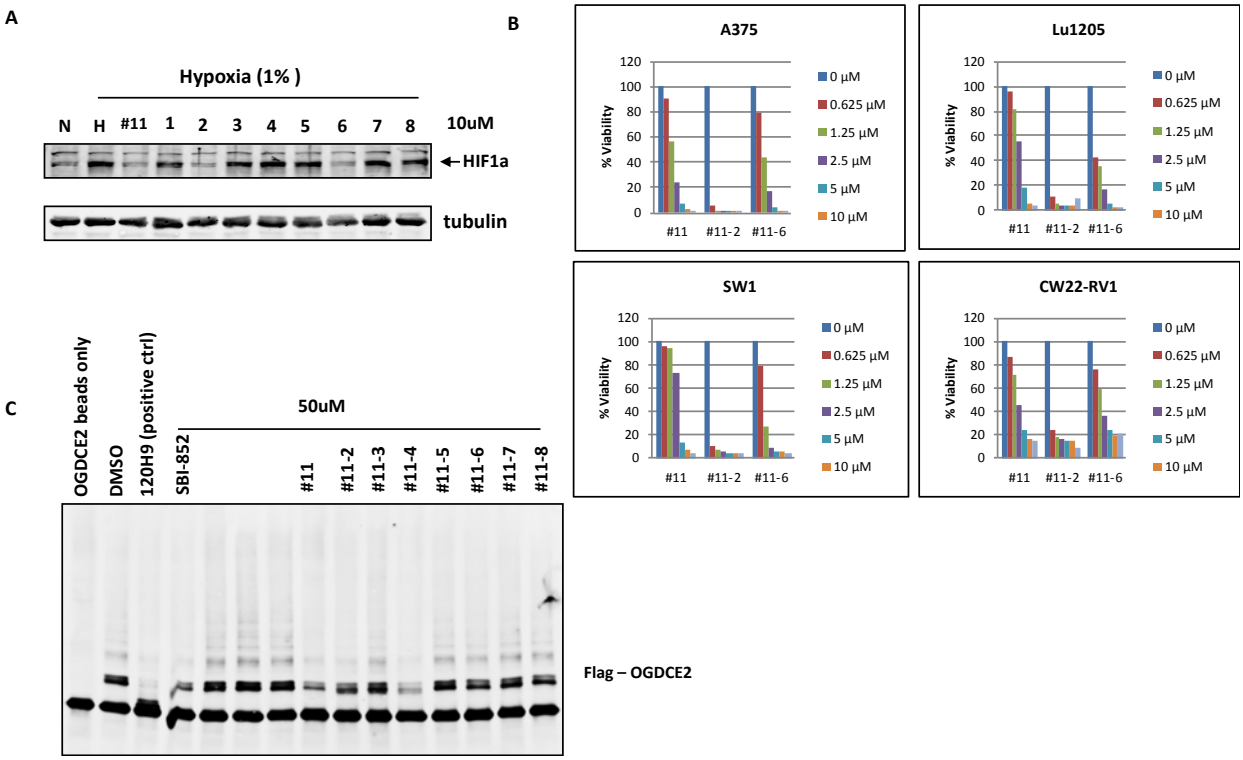



***Figure 1. Effects of compound #11 and its derivatives on HIF1α, proliferation and ubiquitination.*** *(**A**) Different compounds (10 µM) were added to melanoma cells and cultured in 1% oxygen incubator overnight. HIF levels were examined by Western Blot analysis. (**B**) Compound #11 and two derivatives, at the indicated concentrations, were added to different melanoma and prostate cancer cell lines and viability was assessed 72 h after treatment. (**C**) Compound #11 and derivatives were incubated with ODGCE2, a 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. Ubiquitination (smear) was inhibited by compounds 11, 11-2 and 11-4. As positive controls we used Siah2 inhibitory peptides that were designed and used in previous years (120H9 and the compound SBI-852).*

A concern that was raised regarding compound #11 and its derivatives related to their structure that consists of a retinoid structure. We tested whether this component in the structure is important for the activity registered against Siah and found that derivatives that lack the retinoid component were no longer active (Fig. 1 compound 11-8). The concerns that retinoid-like structures may elicit biological activities beyond its effect on Siah ubiquitin ligases, impeded further development of this line of

inhibitors, although, by all means they were superior to any other inhibitor we identified so far for Siah ubiquitin ligases. We discuss future paths to consider, at the end of this progress report.
For these reasons, we initiated additional campaigns to identify possible small molecule Siah inhibitors. To this end, we used an *in silico* approach for the screen of putative Siah inhibitors. We established a collaboration with Dr. Art Cherkov of the Vancouver Prostate Institute in Canada. The *in silico* screen was performed using two complementary approaches. First, we used a structural pocket of Siah that was previously identified to be critical for its inhibition (Fig. 2), in order to test the possibility to identify a small molecule that affect its ubiquitination or ubiquitin ligase activity.

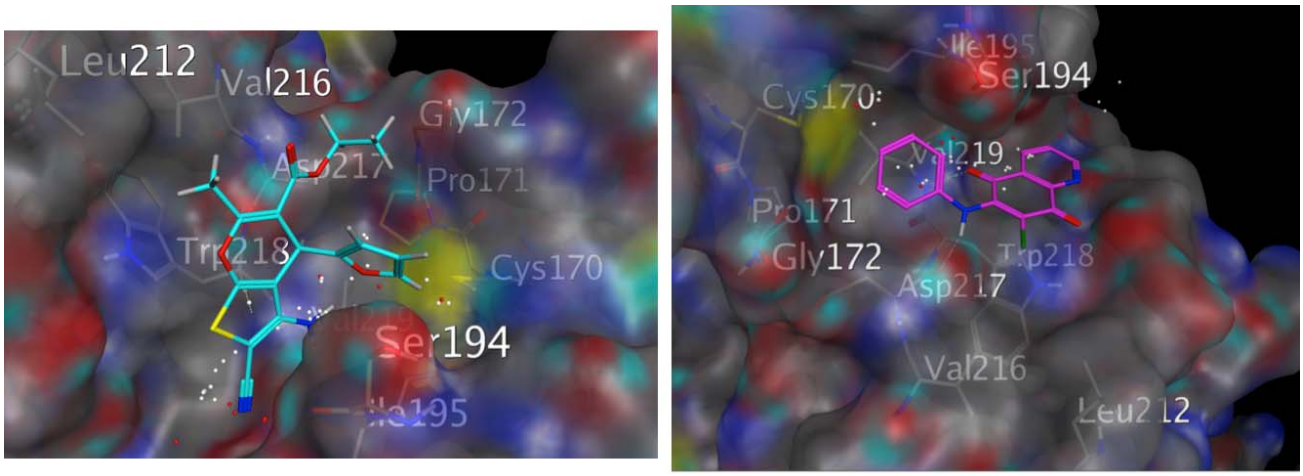



***Figure 2**. In silico, two pockets were used for the screen. In this analysis, the first round of screening identified one clear hit (#55). Possible binding position is hown on the left panel. Predicted binding position of positive hits, exemplified by #55 to the pocket are shown on the right panel. Positive hits which were confirmed biochemically to affect Siah were used as the basis for a second in silico screening campaign in which 29 compounds were selected to further assess the efficacy of the putative inhibitors.*

From over 5 million compounds, 100 were selected based on their affinity to the Siah pocket. Those were purchased and tested for their ability to inhibit Siah2 activity *in vitro* or *in vivo*. Of those, one compound #59 exhibited reproducible activity, measured by means of Siah2 ubiquitin ligase effect on HIF1a stability.

Example of the activity registered with compound #59 is presented in Figure 3. It exhibited good inhibition of HIF1α in cultured cells and a dose dependent inhibition of cell viability as well as potent inhibition of growth in 3D, as determined by colony forming efficiency (CFE) (Fig. 3). *In vitro*, it was also capable of inhibiting Siah2 ubiquitin ligase activity, although, not as efficiently as found for compound #11 (Fig. 3).

In order to further determine the specificity of these compounds for Siah, given that HIF1α was a major target of our screening campaign, we designed another approach to define the effect of Siah inhibitors on the HIF1α axis. To this end, we focused on PHD (prolyl hydroxylases, which are HIF1$\alpha$ regulators). PHD1,2,3 modify HIF1α on prolines by way of hydroxylation, which is a pre-requisite for HIF1α association with its ubiquitin ligase pVHL. If the modification is inhibited (or PHD is inhibited), HIF1α is no longer associated with pVHL enabling its stability. Thus, pharmacological inhibitors for PHD, which were developed and reached clinical trials, are effective modulators of HIF, causing its upregulation. For us, these inhibitors presented a valuable tool, since they phenocopy the effect of Siah ubiquitin ligases. Siah effect on HIF1α stability is mediated by its ubiquitin dependent degradation of PHD1 and PHD3. Inhibition of Siah therefore increases PHD levels and reduces HIF1α stability. Hence, we could test whether PHD inhibitors can overcome the effect of Siah inhibition, and if so, this will confirm that the pathway targeted by Siah inhibitors is indeed specific to the Siah–PHD–HIF axis. To do so, we used luciferase reporter, which monitors HIF1α transcriptional activity, namely HRE-Luc. This commonly used marker is capable of registering transcriptional activities of HIF1α, thereby representing HIF1α

availability. FG4592 (a PHD inhibitor) was added to cells concomitant with the addition of Siah inhibitor, and the level of HRE-Luc was measured 24 h later. As shown, FG4592 (FG) effectively increased HRE-Luc activity (since inhibition of PHD increases HIF) and such increase was effectively attenuated by Siah inhibitors (Fig. 4 upper panel). Here again, inhibitor #11 elicited the most pronounced inhibition, which was dose dependent. Compounds #67 and SBI-852 (an earlier compound identified in our project) were equally potent, whereby #59 was less effective in affecting HIF1α levels. These results further demonstrated the superior effect of #11 on Siah, substantiating its effect is specific, along Siah–PHD–HIF regulatory axis.

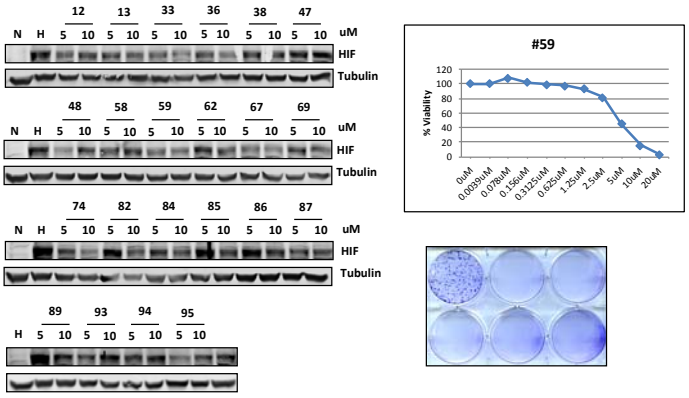
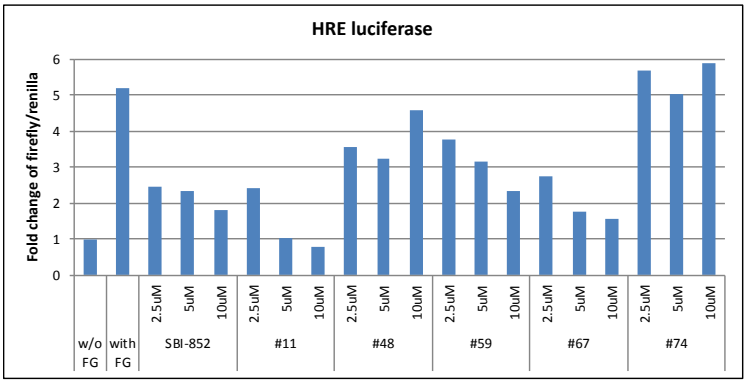



***Figure 3. Effect of compound #59 on HIF1α, proliferation and ubiquitination.*** *(**A**) Selected compounds (5 uM, 10 μM) from in silico-based screen were added to prostate tumor cells and cultured in 1% oxygen incubator overnight. HIF levels were examined by Western Blot analysis. (**B**) Compound #59, at the indicated concentrations, was added to prostate tumor cell line. Viability was assessed 72 h and colonies were stained 10 days after treatment. Upper panel depicts quantification of CFE shown on the lower panel (**C**) Compound #59 was incubated with Siah substrate OGDCE2 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.*

Another approach we undertook to determine the effect of Siah inhibitors was to compare their effect in cells that are Siah2 KO and Siah2 WT. Granted, the cells used in this assessment are MEF (mouse embryo fibroblasts) and therefore may not properly present the biology of the prostate tumor or other transformed cells. The purpose of this assessment was to determine whether the compounds will be less effective in cells lacking Siah2, given their effect is expected to require Siah2. The results revealed mild changes for compound #59, which was less effective in reducing cell viability in Siah2 KO MEFs (Fig. 4 lower panel)



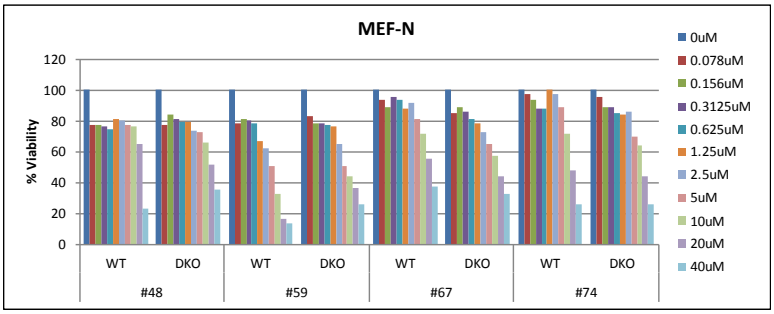




***Figure 4.*** *(**Upper**) Tumor cells were transfected with HRE-luciferase reporter overnight and treated with different compounds at indicated concentration, with or without 10 uM of PHD inhibitor FG-4592. Cells were then lysed and subjected to firefly and renilla luciferase assay. (**Lower**) Selected compounds, at the indicated concentrations, were added to MEF Siah WT and Siah1/Siah2 DKO cells. Viability was assessed 72 h after treatment.*

Given the promising results in culture, we advanced our work to *in vivo* models, both in our lab, and in our collaborators to this funding, Drs. Gleave and Bhowmick. Since we were interested to explore the effect of these inhibitors in immune competent mice, we used a melanoma model of SW1, which we can grow in C3H mice. Addition of #11 to mice one week after administration of SW1 melanoma cells revealed inhibition of melanoma growth over a 5-week period. The degree of inhibition is limited (~30%)

probably due to the limited bioavailability of the compound used. Since #11 was so effective in culture and *in vitro*, further assessment *in vivo* will require adjustments to improve pharmacokinetic properties.

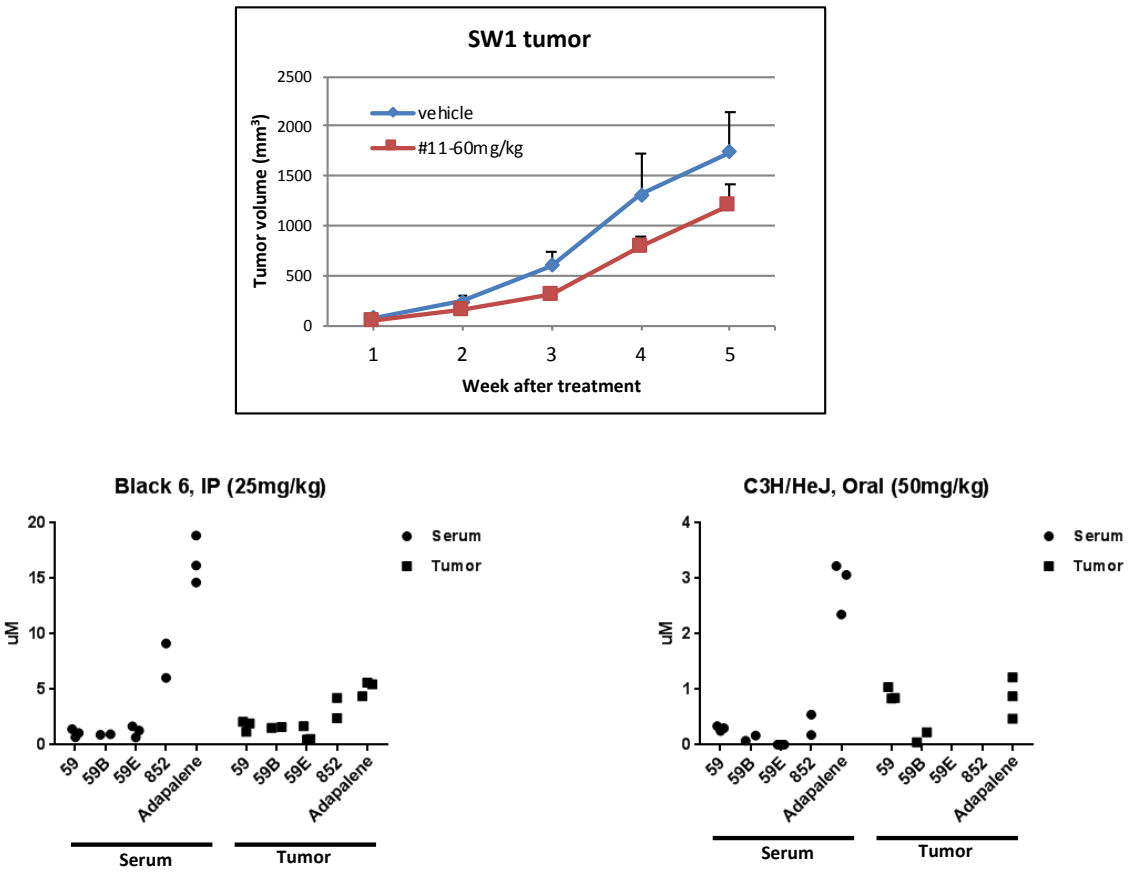



***Figure 5***. (***Upper***) *SW1 mouse melanoma cells were injected subcutaneously (0.5 × $10^6$) into the flank of C3H/HeJ mice and allowed to form established tumors. Once tumors reached approximately 150 $mm^3$, mice were dosed with compound #11 by oral gavage every day (60mg/kg). Tumor size was measured at the indicated time points. (**Lower**). YUMM1.7 and SW1 mouse melanoma cells were injected subcutaneously (0.5 × $10^6$) into the flank of C57Bl/6J (lower left) and C3H/HeJ (lower right) mice respectively. When tumor sizes reached about 300 $mm^3$, compound #59, two derivatives #59B and 59E, SBI-852 and adapalene (aka #11) was administered by intraperitoneal injection (25 mg/kg), or oral gavage (50 mg/kg). Plasma and tumors were collected after 2 hours and analyzed by LC/MS/MS to determine compound levels.*

Lastly, we also continued efforts with the generation of new Siah2 peptides, in collaboration with Drs. Parang Keykavous and Tiwari Rakesh of UC Irvine. New peptides that were generated were tested in culture for their ability to affect HIF1α expression and PHD3 activity. While some of the peptides exhibited some activity, they did not exceed the level of inhibition seen for earlier versions of these peptides. The balance between covalent binding of the peptides to Siah and their ability to penetrate cell membranes effectively could not be achieved in any of the more recent peptides, result that led us to abort this line of investigation.

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

We extended 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. Results presented in earlier section of this report.

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

We performed an additional screening campaign using *in silico* drug screening led to the identification and characterization of additional small molecules, as described above.

**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)

Compound #11 was produced in large quantities and distributed to Drs. Bhowmick and Gleave for *in vivo* assessment, as outlined below.

**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)

This task was fully completed and reported on in previous years.

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

This task was fully completed and reported on in previous years.

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

This task was fully completed and reported on in previous years.

**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 performed these studies using the newly identified Siah inhibitor as outlined above and below.

**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.

**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)

During the year of 2017, Dr. Gleave's lab conducted two animal studies to evaluate if Siah2 inhibition can delay progression of castration resistant disease in the Shionogi model (a model in which Siah2 is increased post castration).

Aim I. To evaluate if inhibition of Siah2 delays progression to castration resistance in Shionogi model. ---100% fulfilled in year of 2017.

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

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. They tested another two 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 antibodies 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.

Ib. Dr. Gleave's lab selected to investigate if a Siah2-inhibiting compound 852 affects repression and recurrence of Shionogi tumor after castration. ----100% fulfilled (prior years).

We conducted the animal work according to the proposal and analysed tumor growth upon the compound 852 treatment. As shown in Figure 8A (below), 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 40 mg/kg, 3 doses/week for about 4 weeks) (Figure 8B below).

Conclusions: The compound SBI-852 didn't potently repress SIAH2 under the conditions tested in our *in vivo* model.

Ic. Dr. Gleave's lab selected to investigate if a Siah2-inhibiting peptide 130B3 affects repression and recurrence of Shionogi tumor after castration. ---- 100% fulfilled.

In the previous animal study, the Siah2 inhibiting peptide 130B3 showed a trend of delaying the growth of Shionogi tumor, but statistical analysis did not reach significance. In order to further confirm if Siah2 inhibition by 130B3 may retard tumor progression, we conducted a 2$^{nd}$ animal study with 130B3 with two major changes:

1) We recruited 15 mice per group instead of 10, which provided more animals by the end points for statistical analysis.
2) We started the 130B3 treatment DAILY for the first 5 days (instead of 3 dose/week) right after the castration and then followed with 3 dose/week until the sacrifice date. This is because Siah2 protein level was induced as early as the 3$^{rd}$ day after the castration, therefore daily injection right after the castration during the first week may provide better repression effect.

*Results and Conclusions:* The Shionogi tumors responded to the castration as expected: tumors shrank after the castration and then recurred, developing into castration resistant disease. However, treatment with 130B3 did not delay tumor progression in this study (Fig. 6A), probably because the 130B3 only slightly repressed Siah2 protein levels (Fig. 6B). More potent inhibition approaches targeting Siah2 may be explored in future instead of 130B3.

A

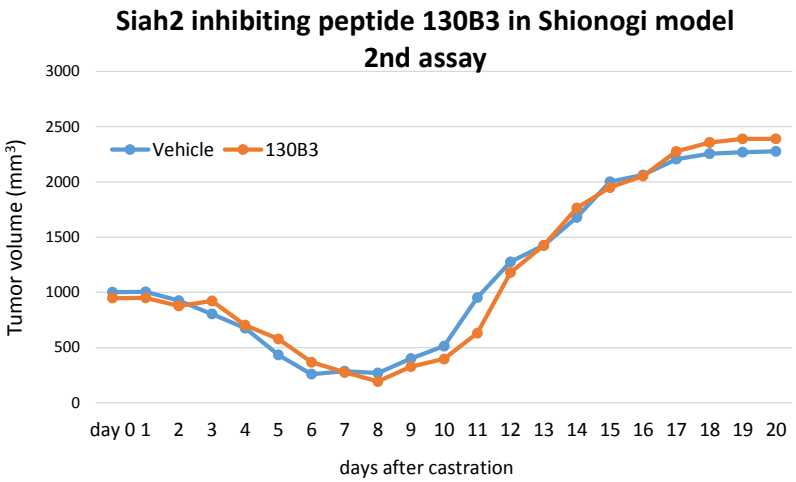
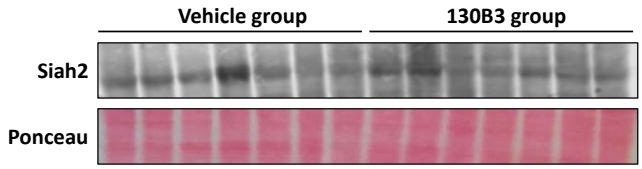



***Figure 6. Effects of Siah2 inhibiting peptide 130B3 on Shionogi tumor progression after castration****. 5x10$^6$ of TD-2 cells were injected subcutaneously into DD/S nude mice. When tumors reached 500–1000 mm$^3$ (2 to 3 weeks after injections) mice were castrated under anesthesia and randomly entered groups for vehicle and 10 mg/kg of 130B3. 130B treatments were started the day after castration with i.v. injection, daily for the first 5 days and then followed with 3 doses/week until the sacrifice date. (A) Tumor volumes were shown in the growth curve. (B) Siah2 protein levels were investigated with Western Blot in the tumor tissue protein lysates.*

B



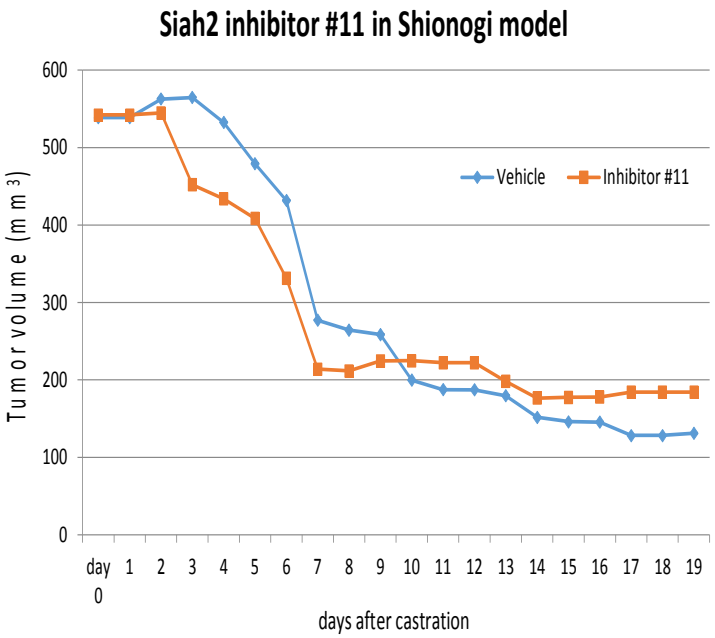

Id. Dr. Gleave's lab selected to investigate if a new Siah2 inhibitor #11 affects regression and recurrence of Shionogii tumor after castration. --- 100% fulfilled in year of 2017.

A new Siah2 inhibitor #11 was tested in the Shionogi tumor model to see if it delayed development of castration resistant disease.

*Procedure:* The inhibitor #11 was given to the mice at the dose of 60 mg/kg body weight, as oral gavage daily starting right after the castration and continued until the sacrifice day. The vehicle was applied as the same manner as the inhibitor.

*Results and Conclusions:* Treatment with inhibitor #11 induced severe toxicity in the animal such as reduced mobility and significant body weight loss; the vehicle itself (started with 300 ul and then reduced to 80 ul) also had mild toxicity effects. We have to terminate mice due to body weight loss (>20%). The tumors shrank after castration but didn't recur probably due to the toxicity effects and poor healthy condition of the mice (Fig. 7). This experiment did not provide a clear answer if the inhibitor #11 can delay progression of the disease due to toxicity effects.

***Figure 7. Effects of Siah2 inhibitor #11 on Shionogi tumor progression after castration.*** *$5x10^6$ of TD-2 cells were injected subcutaneously into DD/S nude mice. When tumors reached 500–1000 $mm^3$ (2 to 3 weeks after injection) mice were castrated under anaesthesia and randomly entered groups for vehicle and 60 mg/kg of #11. #11 treatments were started the day after castration with oral gavage daily until the sacrifice date. Tumor volumes were shown in the growth curve.*




Aim II. To evaluate if inhibition of Siah2 with an inhibitory peptide can inhibit growth of NEPC in PDX LTL352 and LTL331R models. --- 100% fulfilled in prior years.

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

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

**Summary of the project:**

1. In the year of 2015:
   1) We monitored Siah2 levels in the Shionogi model post castration and found out that both mRNA and protein levels of Siah2 were induced as early as 3 days after castration.
   2) We monitored Siah2 levels in the PDX tumors and found out that Siah2 protein levels were enhanced in NEPC tumors comparing to the adenocarcinomas.
   3) We started the *in vivo* assay using the Siah2 inhibiting compound 852 and Siah2 inhibiting peptide 130B3 in the Shionogi model.
   4) We started the *in vivo* assay using 130B3 in the PDX model.
2. In the year of 2016:
   1) We finished the inhibitor compound 852 assay in the Shionogi model but found no effects on repressing Siah2 protein level as well as the tumor progression.
   2) We finished the inhibiting peptide 130B3 assay in the Shionogi model. 130B3 slightly repressed Siah2 protein and showed a trend to delay the disease progression but with no statistically significant. We decided to repeat this assay.
   3) We finished the inhibiting peptide 130B3 assay in the PDX model. The daily i.v. injection was technically challenging with these animal and we didn't see efficient repression on Siah2.
3. In the year of 2017:
   1) We repeated the 130B3 assay in the Shionogi model with more animals each group and earlier treatment starting time point. 130B3 slightly repressed Saih2 protein but showed no effects on tumor progression after castration.

2) We tested a new Siah2 inhibitor #11 in the Shionogi model. Severe toxicity occurred and the animals were terminated due to body weight loss (> 20%). There is no clear answer if the inhibitor #11 may retard the tumor progression.

**Conclusions:**

1. Siah2 is a castration-induced and NEPC-associated protein in the Shionogi and PDX models.
2. The Shionogi and PDX models provide appropriate *in vivo* platforms to study if repression of Siah2 may retard the development of castration resistant diseases.
3. More potent inhibitors targeting Siah 2 in the *in vivo* models are required.
4. Better approaches to monitor Siah2 levels and activities in the *in vivo* models are required.

**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 focuses on rigorous evaluation of novel inhibitors of the ubiquitin ligases Siah1/2 - a major pathway involved in prostate cancer (PCa) castrate resistance (CRPC). Through DOD support we were able demonstrate that both Siah antagonists generated (SBI646852 and 133B3) had effects on the differentiation state of the prostate cancer models tested. The primary *in vivo* model system tested was the tissue recombination orthotopic xenograft including primary human prostate cancer associated fibroblasts with CWR22Rv1 epithelia. This castrate resistant prostate cancer (CRPC) model was used extensively to test the Siah antagonists alone and in combination with androgen axis inhibition (enzalutamide and/or castration). As abiraterone (androgen synthesis antagonist) does not have the same effects in mouse models as in men, castration was used instead. Enzalutamide is a second-generation androgen receptor (AR) inhibitor. The CRPC model system used is partially effected by castration, in terms of reduction in tumor size; but there is little difference in tumor size when castration+enzalutamide is combined, compared to untreated mice. This phenomenon is observed in men that develop enzalutamide addiction. Androgen axis inhibition is recognized to promote neuroendocrine differentiation of prostate adenocarcinoma (PCa) as a means of therapeutic resistance and even metastatic progression. We found that SBI646852 had little effect on the gross tumor volume in the presence or absence of castration or castration+enzalutamide. But SBI646852 seemed to definitively reduce neuroendocrine differentiation of the tumors generated. This encouraging finding led us to examine the next generation Siah antagonist, 133B3, in conjunction with androgen deprivation therapy, with the rational of preventing castrate resistance. We found that 133B3 (10mg/kg) with castration+enzalutamide significantly reduced tumor size, compared to castration+enzalutamide ($n = 12$ per group, $p$ value $< 0.01$). However, 133B3 had little effect on tumor size alone or when combined with castration ($n = 12$). Unfortunately, we lost mice to drug toxicity in the 3-week treatment period in the experiments using 10 mg/kg 133B3. The experiments were repeated 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. Although, drug toxicity was not observed, at the lower 133B3 dose we found no difference in tumor size when combined with castration or castration+enzalutamide ($n = 8$). However, the histologic interrogation demonstrated that even at lower doses, neuroendocrine differentiation was downregulated by the 133B3. This suggested that the mechanism of tumor progression and is neuroendocrine differentiation have different thresholds of Siah activity.

As another mechanism for PCa therapeutic resistance involve the expression of AR splice variants that are missing the ligand binding domain, the role of 133B3 on their expression. We demonstrated that 133B3 inhibited the expression of human AR-V7 and AR-V132. Interestingly, 133B3 was also able to downregulate the expression of the RNA binding protein RBM38 we have found to be central to AR splice variant formation.

In light of our finding that Siah knockdown downregulated prosta-sphere formation we tested the role of 133B3 on metastasis progression. Intra-cardiac injection of luciferase-expressing $ARCaP_M$ cells is recognized to result in both bone and visceral metastasis in a period of 4 weeks. As $ARCaP_M$ have very low AR expression, we monitored metastasis formation by longitudinal bioluminescence in the presence of vehicle or 133B3 (in the absence of androgen deprivation agents). Interestingly, there was a trend (not statistically different) having reduced metastatic growth with 133B3, overall survival was improved (p value < 0.01, Fig. 8).

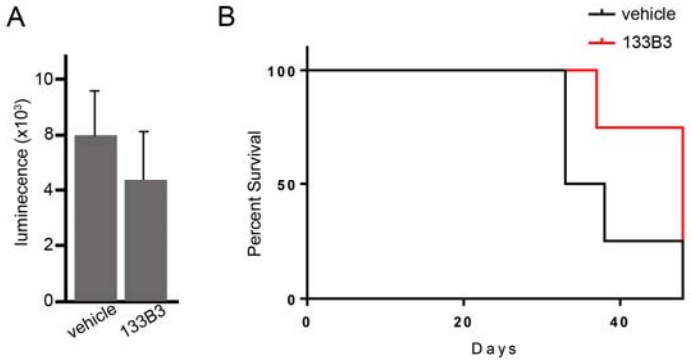



***Figure 8. Testing the role of 133B3 on PCa metastatic progression.*** *(**A**) Bioluminescence signal from intra-cardiac injected $ARCaP_M$ cells was quantitated. (**B**) Overall survival of the mice administered 133B3 was improved, compared to vehicle (p value < 0.01).*

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

We performed studies using the most recently identified Siah inhibitor, as detailed in Drs. Bhowmick and Gleave reports.

**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 performed this task as outlined in Dr. Gleave report

**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 performed these studiesin collaboration with Drs Gleave and Bhowmick reports of previous and last year.

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

The Sanford Burnham Prebys Medical Discovery Institute's (SBP) Graduate School of Biomedical Sciences (GSBS) oversees and coordinates an annual individual development planning (IDP) process for all graduate students in the SBP GSBS program. The focus of the IDP process within GSBS is the development of the educational pathway of the student through identification of the skills, knowledge, and accomplishments that will be necessary for the student to obtain a PhD. degree; and identification of educational and professional development opportunities that are available for the student to obtain the necessary skills and knowledge. GSBS provides guidance and advising to both students and PIs throughout the student's education with respect to developing IDPs and preparing for a successful transition to the next career level post graduation.
The SBP GSBS IDP process includes two components:

> **Student Mentor Annual Reports**. Each year students are required to submit an annual progress report in collaboration with their mentor. This report focuses on the educational goals accomplished through the past school year, highlights the scientific research progress and other

accomplishments made by the students, and outlines an academic and research plan for the following year. Students and their mentor complete this form together and each complete sections providing feedback on the topics above. These reports are reviewed by the Graduate Program Executive Committee (GPEC) each year.

**Annual Thesis Committee Meetings**. Beginning in year two of studies, students are required to assemble their Thesis Committee for an annual meeting to be held between June – November of each year. At these meetings, the student outlines their current specific aims for their thesis project, reports progress made in the previous year and outlines a plan for the future of the project. The thesis committee members provide the student feedback and guidance on the progression of the research project and may suggest additional coursework or training if needed. At the completion of the meeting, the student submits a report signed by the faculty mentor containing a summary of the work they presented, the committee's feedback and plans for continuance to the Graduate Office. This report is then reviewed by GPEC.

Lisa Elmen, a graduate student participated in the above program.

**How were the results disseminated to the communities of interest?**

Nothing to report.

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

Nothing to report.

**4. IMPACT**

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. At present, 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.**

Nothing to report.

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

Nothing to report.

**Changes that have a significant impact on expenditures.**

Nothing to report.

**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**

Nothing to report.

**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 |

| | |
|---|---|
| Name: | Robert Arbecky |
| Project Role: | CPCCG, Associate Director |
| Researcher Identifier: | |
| Nearest person month worked: | 2 |
| Contributions to Project: | Mr. Arbecky contributed to the screen of small molecule inhibitors for Siah which were performed at the CPCCG |
| Funding Support: | N/A |

| | |
|---|---|
| Name: | Gregory Cadwell |
| Project Role: | Lab Manager for Robert Liddington's Lab |
| Researcher Identifier: | |
| Nearest person month worked: | 1 |

| | |
|---|---|
| Contributions to Project: | Mr. Cadwell has prepared purified Siah protein using bacculovirus systems, which were used in our thermal shift assays, screening for Siah inhibitors |
| Funding Support: | N/A |

| | |
|---|---|
| Name: | Lisa Elmen |
| Project Role: | Graduate Student |
| Researcher Identifier: | |
| Nearest person month worked: | 1 |
| Contributions to Project: | Mrs. Elmen has provided reagents for studying the effect on Siah inhibitors for in vitro systmes. |
| Funding Support: | N/A |

**Gleave Lab**

| | |
|---|---|
| Name: | Dr. 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 for the protocol preparation and the proposal conduction. Dr. Zhang has been performing animal work together with the animal staff at Vancouver Prostate Centre and is in charge of the data analysis and report preparation. |
| Funding Support: | |

| | |
|---|---|
| Name: | Dr. 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: | |

| | |
|---|---|
| Name: | Ms. 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: | |

| | |
|---|---|
| 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: | |

| | |
|---|---|
| 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, figure preparation, etc.) |
| Funding Support: | DoD |

**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 |

**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**

Nothing to report.

**Martin Gleave, Partnering PI (Vancouver Prostate Centre)**

Nothing to report.

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

Grants that have ended:

R01 CA108646 (Bhowmick) 08/01/04 – 07/30/17 1.8 calendar (15%)
NIH/NCI
*TGF-ß Signals in Prostate Stromal-Epithelial Interactions*
Goal: The goal of this project was to specifically identify the TGF-ß-mediated signals in the stroma that mediate prostate androgen responsiveness.

1U01CA143057 (Bhowmick/Guise) 09/01/10 – 03/31/17 0.6 calendar (5%)
NIH/NCI
*Differential TGF-Beta Signaling in Bone Microenviroment: Impact on Tumor Growth*
Goal: The proposal tested the hypothesis that, in addition to its effects on tumor cells, TGF-β acts on osteoblasts and osteoclasts to regulate factors that have differing effects on the growth of osteolytic vs. osteoblastic tumor types.

**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 Prebys Medical Discovery 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

N/A