Specific Aims

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive and most common form of pancreatic cancer. Patients with pancreatic cancer have a poor prognosis with a one-year survival of less than 20%. Activating K-Ras mutations occur in over 85% of PDAC cases making it an attractive target for therapy. Inhibitors of the Ras pathway have been FDA approved with the MEK inhibitor, cobimetinib, and PI3K inhibitor, idelalisib, in combination with other therapies being used for the treatment of melanoma and chronic lymphocytic leukemia, respectively. One novel mechanism that may be used to target Ras is the disruption of the plasma membrane localization that is necessary for Ras function. In order for plasma membrane localization to occur, a farnesyl moiety must be added to the cysteine residue of the terminal CAAX motif of Ras, the last three amino acid of the CAAX motif must then be proteolytically cleaved by Ras converting enzyme 1 (RCE1). Finally, isoprenylcysteine carboxyl methyltransferase (ICMT) methylates the C-terminal cysteine to facilitate transport to the plasma membrane. Attempts to target this process with farnesyltransferase inhibitors (FTIs) have failed due to alternate modifications of the CAAX motif in the presence of FTIs and available ICMT inhibitors have limited physical properties for clinical development. However, three RCE1 inhibitors recently derived from previously reported RCE1 inhibitor, NSC1011, have shown promise in disruption of Ras localization in yeast and colon carcinoma cell line, HCT-116. Because of these findings, *I hypothesize that RCE1 inhibition will be effective in preventing oncogenic Ras signaling and therefore an effective therapy for pancreatic ductal adenocarcinoma with activating K-Ras mutations*. To test this hypothesis I propose the following specific aims:

Aim 1: Determine whether inhibition of RCE1 will decrease cell growth and viability of PDAC *in vivo* and *in vitro*. *I hypothesize that inhibition of RCE1 in pancreatic ductal adenocarcinoma will reduce Ras localization to the plasma membrane and Ras-dependent downstream phosphorylation, which will result in decreased pancreatic adenocarcinoma cell viability, migration and growth.* I will treat PDAC cell lines, Panc 02.03 and Panc 04.03, with the RCE1 inhibitors and determine effects to Ras membrane translocation by immunofluorescent microscopy and cell fractionation. I will also study the efficacy of RCE1 inhibition by observing the phosphorylation of the pathway downstream of Ras such as the phosphorylation of MEK, ERK, PI3K and Akt. I will also measure cell viability and growth by trypan blue assay, migration by scratch test and invasion by matrix invasion assays after RCE1 inhibitor treatment. In addition, as a genetic control I will use RCE1 siRNA knockdown to confirm that these findings are specific to RCE1 and not an artifact of the inhibitor. Lastly, I will use these cells in a xenograft mouse model to determine if tumor growth and metastasis is decreased by RCE1 inhibition *in vivo*.

Aim 2: Determine the synergistic effects of targeting RCE1 in combination with inhibitors of downstream effectors in PDAC. Since compensatory mechanisms have been a problem for the efficacy of inhibitors that alter Ras membrane localization, *I hypothesize that RCE1 inhibition will have synergistic effects in combination with downstream inhibitors, such as MEK, Akt or PI3K inhibitors, in pancreatic ductal adenocarcinoma.* In the PDAC cell lines, I will determine synergistic concentrations of the RCE1 inhibitors in combination with MEK, Akt or PI3K inhibitors as determined by viability and cell growth measured by trypan blue assay. I will then treat PDAC cells with the synergistic concentrations of RCE1 inhibitor and MEK, Akt or PI3K inhibitor and determine the effects on migration and invasion by scratch and matrix invasion assays, respectively. Lastly, I will use xenograft mouse models to determine if the inhibition of RCE1 in combination with an inhibitor of a downstream effector will decrease tumor growth and metastasis *in vivo*.

Since Ras mutations are found in ~25% of human tumors, this mechanism may also be applied to many other cancers types, such as prostate and non-small cell lung cancer. Additionally these studies will provide insight into the role Ras membrane localization plays in malignancies.

Significance and Background

Pancreatic ductal adenocarcinoma (PDAC) survival statistics illustrate the need for new therapies.

With a five year survival of about 8%, pancreatic cancer is the fourth leading cause of cancer-related death in the USA (1). Currently, surgical resection is considered the only treatment to cure pancreatic cancer (2). Unfortunately, since pancreatic cancer can progress with few symptoms, most patients are at an advanced stage at diagnosis and resection is only an option for about 10% of cases (3). Chemotherapy and radiotherapy have only been of marginal benefit and only moderately improved patient survival (4). The standard of care for advanced pancreatic cancer since 1997, gemcitabine, was only able to improve the one-year survival rate to 18% and extended the median survival duration to 5.65 months (5). Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, constituting at least 85% of pancreatic cancers (6). PDAC originates from precursor legions, the most common of which is pancreatic intraepithelial neoplasia (PanIN), which arise from ductal epithelial cells (Figure 1) (7). These PanINs are thought to progress from moderate to high-grade dysplasia, and finally to invasive carcinoma (8).

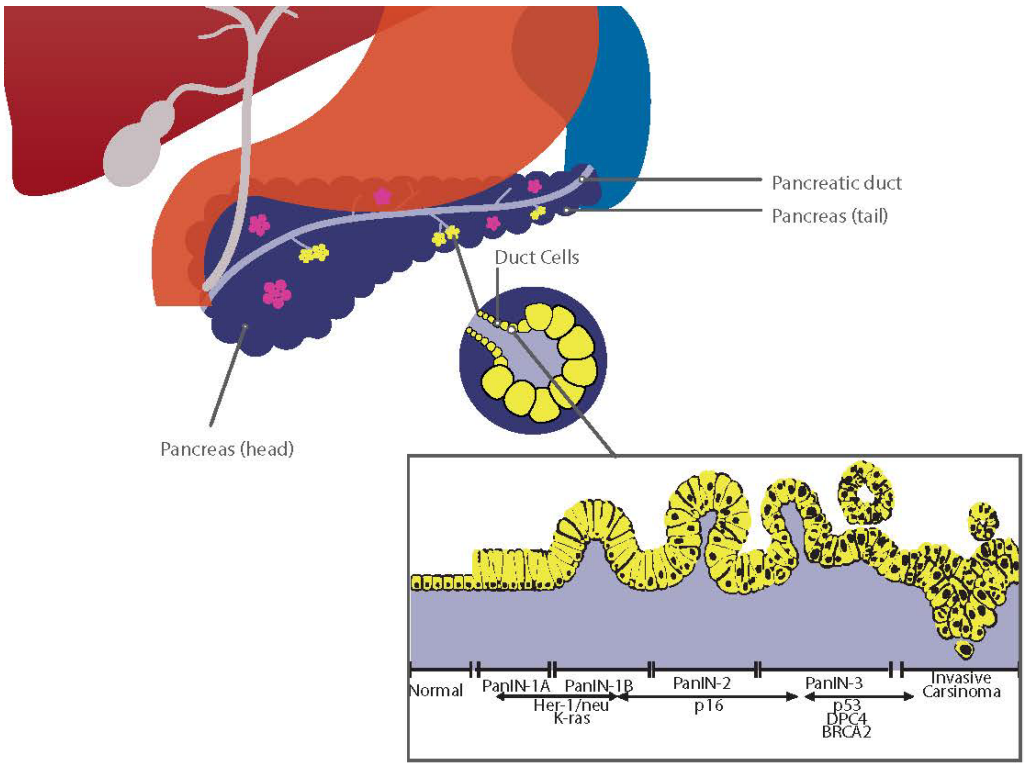

Figure 1: Pancreatic ductal adenocarcinoma (PDAC) arises from pancreatic intraepithelial neoplasia. The pancreatic duct cell, the cell of origin for PDAC, gives rise to pancreatic intraepithelial neoplasia (PanIN) which accumulates genetic mutations as it progresses to invasive carcinoma. Adapted from Hruban RH, Et. Al. (11).

Oncogene dependence in PDAC is a rationale for the use of Ras regulation for treatment.

Though PDAC is a heterogeneous disease with a mean of 50-60 somatic mutations in 12 key pathways, K-Ras mutations are found in over 90% of PDAC patients (9, 10). Activating mutations in K-Ras occur in pancreatic duct lesions and the prevalence of these mutations increase with increasing atypia of the duct lesions, it can therefore be inferred that this is an early genetic event that is key for the progression from neoplasia to invasive carcinoma (11). Since there have been many challenges in inhibiting mutated K-Ras, as it has a unique constitutively active conformation, studies have focused on inhibition of the pathway downstream of Ras, such as Akt, mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) (12). A phase 2 clinical trial of the MAPK/ERK kinase (MEK) inhibitor, selumetinib, in combination with the Food and Drug Administration (FDA) approved epidermal growth factor receptor (EGFR) inhibitor, erlotinib, in previously treated advanced stage PDAC had promising results with a median progression-free survival of 1.9 months and a median overall survival of 7.3 months (13). However, a phase 2 clinical trial to study the effects of selumetinib in combination with the Akt inhibitor, MK-2206, showed no improvement in overall survival (14). Taken together, these clinical studies demonstrated that the inhibition of this pathway is pharmacologically and physiologically possible, however further research is needed to optimize the effective use of these inhibitors in the treatment of pancreatic ductal adenocarcinoma.

Inhibition of the transport to the cell membrane impedes Ras activity.

In addition to inhibiting downstream targets, the transport and localization of Ras to the membrane as a possible target for inhibition has been investigated (15). Ras must associate with the inner plasma membrane to

function, therefore interfering with the transport of Ras to the plasma membrane can suppress Ras activity even if it's constitutively active (16). Ras localization to the cell membrane is a multi-step process (Figure 2). First, Ras undergoes prenylation by farnesyltransferase (FTASE) (Figure 2, A) and subsequently has its terminal AAX motif proteolytically cleaved by Ras converting enzyme 1 (RCE1) (Figure 2, B) before methylation by isoprenylcysteine carboxyl methyltransferase (ICMT) can occur (Figure 2, C) (17). Ras can then be transferred to the recycling endosome or Golgi to be shuttled directly by vesicular transport to the plasma membrane (18). Initial attempt to inhibit this process focused on targeting farnesyltransferase but it was discovered that in the presence of farnesyltransferase inhibitors, Ras is prenylated by geranylgeranyl transferase (GGTASE) (Figure 1, D)(19).

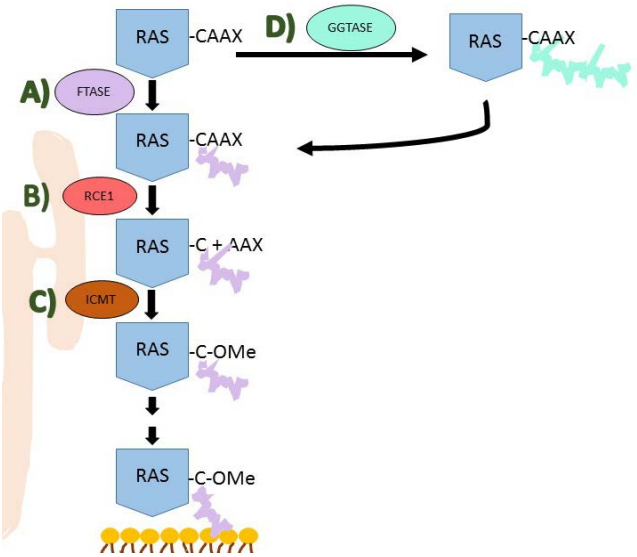

Figure 2: Transport and localization of Ras to the cell membrane requires RCE1, ICMT and farnesylation or geranylgeranylation. Transport to the endoplasmic reticulum (ER) is essential for the removal of the AAX motif, so ICMT can methylate the C-terminal cysteine and facilitate membrane association. Adapted from Papke B and Der CJ (18).

RCE1 inhibition can be used to regulate Ras.

Blockade of RCE1 can cause mis-localization of Ras and reduce oncogenic transformation of cells (20). A recent study has derived novel RCE1 inhibitors from NSC1011, an 8-Hydroxyquioline-based inhibitor of RCE1 (21). Three of which, NSC1011-2, NSC1011-6 and NSC1011-17 (Figure 3), show promise in the selective and effective disruption of Ras localization and activity as seen in yeast and colon carcinoma cell line, HCT-116 (21). These compounds were able to inhibit 50% of CAAX cleavage, as measured by a fluorescence-based proteolysis assay in yeast, at concentrations of about 10 μM and did not induce more than a 20% loss in viability of mammalian cells at concentrations of 25 μM after 20 hours, as measured by CellTiter-Blue assay (21). Additionally, these compounds were able to mis-localize Ras as effectively as knockdown of RCE1 as measured by decrease in plasma membrane association to 20-30% as determined by fluorescent microscopy (21). Lastly, these inhibitors showed specificity for RCE1 over functionally similar Ste24 protease, which mediates zinc-dependent CAAX proteolysis in the maturation of lamin A (22), as demonstrated by the decrease in activity of Ste24 by less than 25% and the decrease in activity of RCE1 by about 60% after 10μM treatment with the inhibitors (21).

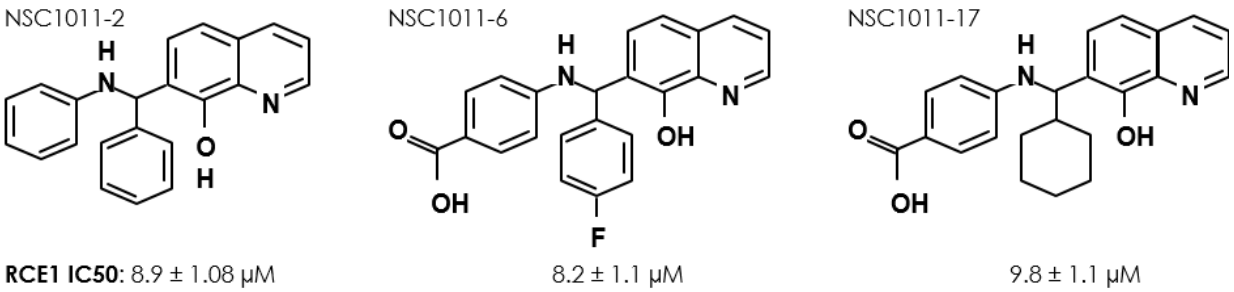

Figure 3: Three novel RCE1 inhibitors recently derived from NSC1011 show promise for the treatment of PDAC. Novel RCE1 inhibitors, NSC1011-2, NSC1011-6 and NSC1011-17, are able to induced 50% inhibition of RCE1 at concentrations less than 10 μM. Adapted from Mohammed I, Et Al. (21)

Understanding the effects of RCE1 inhibition may improve health outcomes for PDAC patients and will provide insight on how to better treat PDAC as well as other diseases with K-Ras mutations.

Research Plan

Aim 1: Determine whether inhibition of RCE1 will decrease cell growth and viability of PDAC *in vivo* and *in vitro*.

Hypothesis/Rationale: Recently the RCE1 inhibitors derived from the RCE1 inhibitor, NSC1011, have been shown to have specific and effective inhibition of RCE1 in yeast and the HCT-116 colon carcinoma cell line (21). It has been previously published that loss of RCE1 prevents the localization of Ras to the cell membrane and reduces oncogenic transformation of cells (20). *I hypothesize the inhibition of RCE1 in pancreatic ductal*

adenocarcinoma will reduce Ras localization to the plasma membrane and Ras-dependent downstream phosphorylation, which will result in decreased pancreatic adenocarcinoma cell viability, migration, invasion and growth.

Approach:

Aim 1.1: Determine molecular effects of RCE1 inhibition on PDAC. To determine the molecular and cellular effects of RCE1 inhibition on PDAC cells, the Panc 02.03 and Panc 04.03 cell lines will be utilized. Both cell lines are commercially available, have been verified to possess K-Ras oncogenes, form tumors in nude or severe combined immunodeficiency (SCID) mice and were harvested in 1995 from the head-of-the-pancreas of pancreatic adenocarcinoma patients, specifically a 64 year old white female and 70 year old white male, respectively. **First, to determine if the RCE1 inhibitors can prevent Ras membrane localization in PDAC cells, I will perform fluorescent microscopy and subcellular fractionation experiments**. For the fluorescent microscopy, cells from both cell lines will be seeded at 60% confluency on coverslips, then treated with the RCE1 inhibitors at the concentration determined to be the IC50 for RCE1 activity determined previously (21) or vehicle control for 24 hours. RCE1 and control siRNA knockdown will be used a genetic control to ensure results are specific for RCE1 and not an artifact of off-target effects of the inhibitor. The cells on the coverslips will be blocked then probed with K-Ras antibody, DAPI nuclear stain and CellBrite plasma membrane stain. The signal will be visualized using fluorescent microscopy and analyzed for co-localization of the Ras antibody to the membrane using Ziess confocal microscope and ZEN imaging software. For the subcellular fractionation, the ultra-centrifugation protocol available through Abcam will be performed on the lysates of 10 million cells from both cell line treated with RCE1 inhibitors at the concentration determined to be the IC50 for RCE1 activity determined previously (21) or vehicle control for 24 hours to separate the nuclear cytoplasmic, mitochondrial and membrane fractions. RCE1 and control siRNA knockdown will be used a genetic control to ensure results are specific for RCE1 and not an artifact of off-target effects of the inhibitor. Fractions will be run by SDS-PAGE then transferred to a PVDF membrane. The membrane will be blocked then probed with Ras, GAPDH, cytochrome c, laminin A/C and Na/K ATPase to determine the separation of the cytosolic, mitochondria, nuclear and membrane faction respectively. The signal will be visualized using enhanced chemiluminescence (ECL) reagents then quantified by densitometry using ImageJ software. **To determine if the RCE1 inhibitors can prevent Ras-dependent phosphorylation of downstream signaling pathways, I will utilize immunoblotting techniques** to analyze phosphorylated phosphatidylinositol-3-kinase (PI3K), Akt, MEK and ERK. I will treat 5 million of each cells treated with RCE1 inhibitors at the IC50 concentration for RCE1 activity determined previously (21) or vehicle control for 24 hours. RCE1 and control siRNA knockdown will be used a genetic control to ensure results are specific for RCE1 and not an artifact of off-target effects of the inhibitor. The cells will then be lysed and total protein concentrations will be determined by Bradford assay. Equal total protein amounts will be run by SDS-PAGE and transferred to a PVDF membrane. The membrane will be blocked and probed for phosphorylated and total PI3K, Akt, MEK and ERK, as well as actin to control for loading. The signal will be observed using ECL reagents then quantified by densitometry using ImageJ software. All results will be analyzed for statistical significance using GraphPad Prism software.

Aim 1.2: Determine cellular effects of RCE1 inhibition on PDAC. To determine the cellular effects of RCE1 inhibition, I will perform cell viability, cell growth, cell migration and cell invasion assays, as well as *in vivo* studies in mouse xenograft models to determine the effects on tumor burden, metastasis and survival. **First, to determine how RCE1 inhibition affects viability and growth in PDAC cells, I will conduct trypan blue exclusion tests and cell count assays.** I will treat 500,000 cells from each cell line with the RCE1 inhibitors at the concentration determined to be the IC50 for RCE1 activity determined previously (21) or vehicle control for 24 hours then viability and cell count will be measured by trypan blue using the Vi-Cell cell counter. The cell growth will be determined from the cell count compared to control. RCE1 and control siRNA knockdown will be used as a genetic control to ensure results are specific for RCE1 and not an artifact of off-target effects of the inhibitor. **Next, to determine migration and invasion, I will perform scratch tests and matrix invasion assays.** Migration will be measured by seeding a confluent monolayer of cell lines treated with the RCE1 inhibitors at the concentration determined to be the IC50 for RCE1 activity determined previously (21) or vehicle control for 24 hours in a tissue culture dish. The surface of the dish will be scratched with a pipette tip and images of the dish will be taken by IncuCyte every 4 hours for 48 hours. RCE1 and control siRNA knockdown will be used as a genetic control to ensure results are specific for RCE1 and not an artifact of off-target effects of the inhibitor. Distance between the sides of the scratch will be analyzed by Media Cybernetics Image Pro-Plus software. Invasiveness will be assessed by seeding cells from each cell line treated with the RCE1 inhibitors at the concentration determined to be the IC50 for RCE1 activity determined previously (21) or vehicle control for

24 hours at 70% confluency on an upper compartment containing cell permeable membrane coated with basement membrane extract at low, medium and high density available through R&D Systems, cells that degrade the matrix and migrate through the membrane will be stained and counted after 48 hours. RCE1 and control siRNA knockdown will be used as a genetic control to ensure results are specific for RCE1 and not an artifact of off-target effects of the inhibitor. **Lastly, to determine the effects of RCE1 inhibition on tumor burden, metastasis and survival, I will use a xenograft mouse model.** To measure tumor burden and metastasis by luciferin bioluminescence, I will transduce Panc 02.03 and Panc 04.03 cells with a luciferase tag. This will allow for non-invasive imaging of the cells using IVIS 100 or IVIS Spectrum *in vivo* imaging systems provided by the small animal imaging facility at MD Anderson Cancer Center. Dose response assays will be performed on the transduced cells to ensure the vector and luciferase tag have not altered the cells response to the RCE1 inhibitors. For the *in vivo* experiments, a cell line derived orthotopic xenograft athymic nude mouse model will be utilized as this provides essential stromal support. This also contributes an immune system with intact natural killer cells, B-cells, granulocytes, macrophages and dendritic cells that is only lacking in mature T-cells, while still allowing for engraftment (23). Athymic nude mice grouped into 4 arms containing 5 mice each will be injected orthotopically with 200,000 cells from each cell line tagged with luciferase, once the cells have engrafted as determined by luciferin bioluminescence the mice will be injected intravenously with the RCE1 inhibitor that caused the greatest decrease in viability, cell growth, migration and invasion at the concentration determined to be the IC50 for RCE1 activity determined previously (21) or vehicle control every 24 hours (Figure 4). Survival will be assessed as well as tumor burden and metastasis as measured by luciferin bioluminescence every 7 days for 120 days. Animal studies will be performed in accordance with the Animal Care and Use Committee of the University of Texas M.D. Anderson Cancer Center. Animals will be sacrificed once the tumor has reached a diameter of 1.5cm or at signs of distress, such as inability to access food or water or labored respiration.

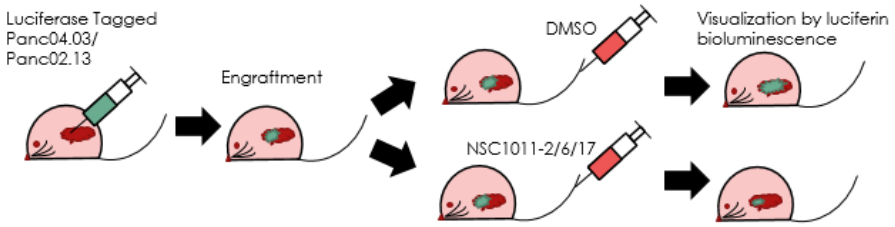

Figure 4: Schematic for animal studies. Athymic nude mice will be injected orthotopically with luciferase tagged PDAC cells. Once the cells have engrafted, the mouse models will be treated with the RCE1 inhibitor which was most effective *in vitro*. Tumor burden and metastasis will be tracked by luciferin bioluminescence every 7 days.

Expected Outcomes/Alternative Approaches:

I expect the use of the novel RCE1 inhibitors and RCE1 knockdown by siRNA will significantly decrease Ras phosphorylation of PI3K, Akt, MEK and ERK, as measured by immunoblot, and Ras localization to the cell membrane as measured by fluorescent microscopy and subcellular fractionation in the cell lines. Additionally, I expect the use of the RCE1 inhibitors and RCE1 knockdown by siRNA will decrease viability and cell growth as measured by trypan blue assay, migration as measured by the scratch migration assay, and invasion as measured by the matrix invasion assay. Lastly, I expect RCE1 inhibition will decrease tumor burden and metastasis as measured by luciferin bioluminescence and increase survival in the athymic mouse models.

As RCE1 inhibition and knockdown have been shown to disrupt Ras localization to the cell membrane (20), it is highly unlikely that there will not be a decrease in co-localization to the membrane in PDAC cells. If this is not seen, the concentrations of inhibitors will be altered as determined by dose response. It is unlikely that a decrease in phosphorylation of PI3K, Akt, MEK and ERK are not seen with RCE1 inhibition or siRNA knockdown if there is a decrease in Ras plasma localization, as Ras activity has been shown to be dependent on plasma membrane localization (16). It is additionally unlikely that there will not be a decrease in viability, cell growth, migration or invasiveness of the PDAC cells with the RCE1 inhibitors or siRNA knockdown if a decrease in the phosphorylation of PI3K, Akt, MEK and ERK is seen as these are key signaling pathways for survival and proliferation. If either is the case however, I will alter the concentrations and time points at which the assays are measured as determined by dose response and an 8 to 120 hour time course with the RCE1 inhibitors and siRNA. If alterations to the concentrations and time point do not produce the expected molecular and cellular effects of RCE1 inhibition, RNASeq and CyTOF will be performed to identify a compensatory mechanism. It is unlikely that I will not see a decrease in tumor burden or metastasis or an increase in survival in athymic nude mice injected orthotopically with PDAC cells then treated with the RCE1 inhibitor that caused the greatest decrease in viability, cell growth, migration and invasion, as these *in vitro* results should be recapitulated in the *in vivo* model. If this is the case however, I will alter the concentration and dosing schedule of the inhibitor injections as determined by pharmacokinetic assays. If the cells cannot be tagged with luciferase without altering

their response to the RCE1 inhibitors, tumor burden and metastasis will be determined postmortem. Alternatively, cell line derived orthotopic xenograft "humanized" NOD/SCID mouse models or PDX-1-Cre, LSL-$Kras^{G12D}$, LSL-$Trp53^{R172H/-}$ (KPC) genetically engineered mouse models (GEMMs) will be used instead of athymic nude mice, if engraftment of PDAC cells does not occur in athymic nude mice or if cell line derived orthotopic xenograft do not resemble the histopathology of human PDAC (23). In addition, the pancreas and metastatic tumors will be harvested to determine the efficacy of RCE1 inhibition *in vivo* as measured by the cleavage of terminal –AAX motif of Ras and/or Ras membrane localization. These tumors can also be used to identify other proteins that may be acting as compensatory mechanisms in the presence of RCE1 inhibition.

Aim 2: Determine the synergistic effects of targeting RCE1 in combination with inhibitors of downstream effectors in PDAC.

Hypothesis/Rationale: Previous attempts to target the Ras pathway often lead to compensatory mechanisms that reduce the effectiveness of monotherapies (19). However, combinations of inhibitors targeting multiple nodes within the same signaling pathway, such as the use of an RCE1 inhibitor in combination with a downstream inhibitor, can reduce the likelihood of this occurring. Inhibitors of downstream effectors, such as MEK, Akt and PI3K, are already FDA approved for the treatment of other cancer or are in clinical trials for the treatment of PDAC. Additionally, by using a combination treatment that work synergistically the concentrations of the inhibitors required can be lowered, limiting off target binding and decreasing side effects. *I hypothesize that RCE1 inhibition will have synergistic effects in combination with inhibitors of downstream effectors, such as MEK, Akt or PI3K, in pancreatic ductal adenocarcinoma.*

Approach:

Aim 2.1: Determine synergistic effects of RCE1 inhibitors in combination with MEK, Akt or PI3K inhibitors. To determine the concentrations at which RCE1 inhibitors have synergistic effects in combination in MEK, Akt or PI3K inhibitors, I will perform combination dose response assays for viability and cell growth in PDAC cells. I will start the dose response with concentrations that are lower and higher than the reported IC50 of each inhibitor, by half log or log increments. As the RCE1 inhibitors were previously reported to have IC50s of about 10 μM (21), the concentrations of RCE1 inhibitors for the dose response will be 1, 3, 10 and 30 μM. The MEK inhibitor, selumetinib, which has been used in clinical trials for PDAC (13, 14) has an IC50 of 14nM according to the Selleckchem technical datasheet and is able to reduce cell phosphorylation of ERK1/2 *in vitro* at concentrations of less than 0.5 μM (24) therefore selumetinib at the concentrations of 0.01, 0.03, 0.1, 0.3 and 1 μM will be used in the dose response. Since the Akt inhibitor, MK-2206, has IC50s for Akt 1, 2, and 3 of 8, 12 and 65 nM, respectively, according to the Selleckchem technical datasheet, and all three isoforms have been shown to play a role in chemoresistance (25) MK-2206 at the concentrations of 3, 10, 30, and 100 nM will be used for the dose response. Lastly, the PI3K inhibitor, idelalisib, currently FDA-approved for the treatment of chronic lymphocytic leukemia, has an IC50 of 2.5 nM for the p110δ subunit of PI3K according to the Selleckchem technical datasheet and has been shown to decrease viability of multiple myeloma patient samples by 50% with no effect to healthy cells at concentrations lower than 20μM (26). Therefore, I will use idelalisib at the concentrations of 0.01, 0.1, 1 and 10 μM for the dose response. I will treat 500,000 cells from each cell line with the RCE1 inhibitor and MEK, Akt or PI3K inhibitor, separately and in combination, at the determined concentrations. The viability and cell growth will be measured by trypan blue using the Vi-Cell cell counter. CalcuSyn will be used to analyze the results for synergistic effects.

Aim 2.2: Evaluate effects of RCE1 inhibition in combination with MEK, Akt or PI3K inhibition on migration, invasion and in vivo tumor burden, metastasis and survival. I will seed a confluent monolayer of each cell line treated with the RCE1 inhibitor determined to have the most synergistic effects or vehicle control in combination with MEK, Akt, or PI3K inhibitor or vehicle control at the synergistic concentrations determined in a tissue culture dish. The surface of the dish will be scratched and the distance between the sides of the scratch will be assessed as described in Aim 1.2. **Next, I will determine the effects of RCE1 inhibition in combination with MEK, Akt or PI3K inhibition on invasiveness by matrix invasion assays.** PDAC cells from each cell line will be treated with the most synergistic RCE1 inhibitor or vehicle control in combination with MEK, Akt, or PI3K inhibitor or vehicle control at the determined synergistic concentrations for 24 hours will be seeded at 70% confluency on an upper compartment containing cell permeable membrane coated with basement membrane extract at low, medium and high density available through R&D Systems, cells that degrade the matrix and migrate through the membrane will be stained and counted after 48 hours and invasiveness will be assess as described in Aim 1.2. Lastly, I will use PDAC cells in an orthotopic xenograft mouse model to determine if tumor growth and metastasis

are decreased *in vivo* after treatment with the synergistic RCE1 inhibitor and MEK, Akt, or PI3K inhibitor determined to have the most synergistic effects. Athymic nude mice will be grouped into 8 arms containing 5 mice each which will be injected orthotopically with 200,000 cells from each cell line tagged with luciferase, once the cells have engrafted as determined by luciferin bioluminescence the mice will be injected with RCE1 or the inhibitor of the downstream effector that had resulted in the best effect *in vitro* at the concentrations determined to have the most synergistic effects, or the combination of both RCE1 inhibitor and downstream effector inhibitor or vehicle control every 24 hours. These concentrations are not to exceed the 120 mg/kg, 100mg/kg and 30mg/kg doses recommended for MK-2206, selumetinib and idelalisib, respectively, according to the Selleckchem technical datasheet. Survival will be assessed as well as tumor burden and metastasis as measured by luciferin bioluminescence, as described in Aim 1.2. Animal studies will be performed in accordance with the Animal Care and Use Committee of the University of Texas M.D. Anderson Cancer Center. Animals will be sacrificed once the tumor has reached a diameter of 1.5cm or at signs of distress, such as inability to access food or water or labored respiration.

Expected Outcomes/Alternative Approaches:

I expect cell viability, cell growth, migration and invasiveness of PDAC cells to decrease when treated with RCE1 inhibitor in combination with MEK, Akt, or PI3K inhibitors. I expect mice orthotopically injected with PDAC cells then treated with RCE1 inhibitor in combination with MEK, Akt, or PI3K inhibitors to have decreased tumor burden and metastasis and increased survival. It is unlikely that there will not be at least an additive effect of using RCE1 inhibitor in combination with MEK, Akt, or PI3K inhibitors, however there is a possibility that no synergistic effects will be seen, as these inhibitors are targeting the same pathway. **In the case that only an additive effect is seen, the inhibitors will be used at their lowest combined concentration that still induces a decrease in viability, cell growth migration and invasion as this should still provide the benefit of reducing the concentration of each inhibitor needed to induce the desired effect.** As discussed in the expected outcomes/alternative approaches of Aim 1, it is unlikely that there will not be a decrease in viability, cell growth, migration or invasiveness of the PDAC cells with combination of the RCE1 inhibitor and MEK, Akt, or PI3K inhibitor, even if no decrease in viability, cell growth, migration or invasiveness is seen with RCE1 inhibitor, as MEK, Akt, or PI3K are key signaling pathways for survival and proliferation. If that is the case however, I will alter the time points at which the assays are measured as determined by an 8 to 120 hour time course with the RCE1 inhibitor and MEK, Akt, or PI3K inhibitor. It is unlikely that I will not see a decrease in tumor burden or metastasis or an increase in survival in athymic nude mice injected orthotopically with PDAC cells then treated with the combination of RCE1 inhibitor and ICMT, MEK, PI3K or MAPK, if there is a decrease in viability, cell growth, migration or invasiveness. If this is the case however, I will alter the dosing schedule of the inhibitor injections as determined by pharmacokinetic assays. As mentioned in the alternative approaches in Aim 1, if the cells cannot be tagged with luciferase without altering their response to the RCE1 inhibitors, tumor burden and metastasis will be determined postmortem. Additionally, as mentioned in the alternative approaches in Aim 1, cell line derived orthotopic xenograft “humanized” NOD/SCID mouse models or PDX-1-Cre, LSL-$Kras^{G12D}$, LSL-$Trp53^{R172H/-}$ (KPC) genetically engineered mouse models (GEMMs) will be used instead of athymic nude mice, if engraftment of PDAC cells does not occur in athymic nude mice or if cell line derived orthotopic xenograft do not resemble the histopathology of human PDAC (23,24). Finally, the pancreas and metastatic tumors can be harvested to determine the efficacy of RCE1 and MEK, Akt or PI3K inhibition *in vivo* and/or to identify compensatory mechanisms, as mentioned in the alternative approaches in Aim 1, if there is no change in survival, tumor burden and/or metastasis.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016; 66(1):7-30.
2. Kamisawa T, Wood LD, Itoi T, Takaori K. Pancreatic Cancer. Lancet. 2016; 388(10039):73-85.
3. Hidalgo M, Cascinu S, Kleeff J, et al. Addressing the challenges of pancreatic cancer: future directions for improving outcomes. Pancreatology. 2015;15(1):8-18.
4. Adamska A, Domenichini A, Falasca M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. Int J Mol Sci. 2017; 18(7).
5. Burris HA, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol. 1997;15(6):2403-13.
6. Conroy T, Bachet JB, Ayav A, et al. Current standards and new innovative approaches for treatment of pancreatic cancer. Eur J Cancer. 2016;57:10-22.

7. Fokas E, O'neill E, Gordon-weeks A, Mukherjee S, Mckenna WG, Muschel RJ. Pancreatic ductal adenocarcinoma: From genetics to biology to radiobiology to oncoimmunology and all the way back to the clinic. Biochim Biophys Acta. 2015;1855(1):61-82.
8. Muniraj T, Jamidar PA, Aslanian HR. Pancreatic cancer: a comprehensive review and update. Dis Mon. 2013;59(11):368-402.
9. Falasca M, Kim M, Casari I. Pancreatic cancer: Current research and future directions. Biochim Biophys Acta. 2016;1865(2):123-32.
10. Chiaravalli M, Reni M, O'reilly EM. Pancreatic ductal adenocarcinoma: State-of-the-art 2017 and new therapeutic strategies. Cancer Treat Rev. 2017;60:32-43.
11. Hruban RH, Goggins M, Parsons J, Kern SE. Progression model for pancreatic cancer. Clin Cancer Res. 2000;6(8):2969-72.
12. Dunne RF, Hezel AF. Genetics and Biology of Pancreatic Ductal Adenocarcinoma. Hematol Oncol Clin North Am. 2015;29(4):595-608.
13. Mcnally LR, Mezera M, Morgan DE, et al. Current and Emerging Clinical Applications of Multispectral Optoacoustic Tomography (MSOT) in Oncology. Clin Cancer Res. 2016;
14. Chung V, Mcdonough S, Philip PA, et al. Effect of Selumetinib and MK-2206 vs Oxaliplatin and Fluorouracil in Patients With Metastatic Pancreatic Cancer After Prior Therapy: SWOG S1115 Study Randomized Clinical Trial. JAMA Oncol. 2017;3(4):516-522.Oettle H. Progress in the knowledge and treatment of advanced pancreatic cancer: from benchside to bedside. Cancer Treat Rev. 2014;40(9):1039-47.
15. Paulson AS, Tran cao HS, Tempero MA, Lowy AM. Therapeutic advances in pancreatic cancer. Gastroenterology. 2013;144(6):1316-26.
16. Cox AD, Der CJ, Philips MR. Targeting RAS Membrane Association: Back to the Future for Anti-RAS Drug Discovery?. Clin Cancer Res. 2015;21(8):1819-27.
17. Simanshu DK, Nissley DV, Mccormick F. RAS Proteins and Their Regulators in Human Disease. Cell. 2017;170(1):17-33.
18. Papke B, Der CJ. Drugging RAS: Know the enemy. Science. 2017;355(6330):1158-1163.
19. Vasan N, Boyer JL, Herbst RS. A RAS renaissance: emerging targeted therapies for KRAS-mutated non-small cell lung cancer. Clin Cancer Res. 2014;20(15):3921-30.
20. Wang Y, Kaiser CE, Frett B, Li HY. Targeting mutant KRAS for anticancer therapeutics: a review of novel small molecule modulators. J Med Chem. 2013;56(13):5219-30.
21. Mohammed I, Hampton SE, Ashall L, et al. 8-Hydroxyquinoline-based inhibitors of the RCE1 protease disrupt Ras membrane localization in human cells. Bioorg Med Chem. 2016;24(2):160-78.
22. Tam A, Schmidt WK, Michaelis S. The multispanning membrane protein Ste24p catalyzes CAAX proteolysis and NH2-terminal processing of the yeast a-factor precursor. J Biol Chem. 2001;276(50):46798-806.
23. Ponz-sarvise M, Tuveson DA, Yu KH. Mouse Models of Pancreatic Ductal Adenocarcinoma. Hematol Oncol Clin North Am. 2015;29(4):609-17.
24. Huynh H, Soo KC, Chow PK, Tran E. Targeted inhibition of the extracellular signal-regulated kinase kinase pathway with AZD6244 (ARRY-142886) in the treatment of hepatocellular carcinoma. Mol Cancer Ther. 2007;6(1):138-46.
25. Gdowski A, Panchoo M, Treuren TV, Basu A. Emerging therapeutics for targeting Akt in cancer. Front Biosci (Landmark Ed). 2016;21:757-68.
26. Ikeda H, Hideshima T, Fulciniti M, et al. PI3K/p110{delta} is a novel therapeutic target in multiple myeloma. Blood. 2010;116(9):1460-8.