AWARD NUMBER: W81XWH-21-1-0901

TITLE: Modulating Neural Signaling in the Treatment of Pancreatic Cancer

PRINCIPAL INVESTIGATOR: Dr. Timothy Wang

CONTRACTING ORGANIZATION: Columbia University Irving Medical Center; 1130 St. Nicholas Ave. Rm 901, New York, NY 10032

REPORT DATE: OCTOBER 2022

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Development 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 2022	ANNUAL	15SEPT2021 - 14SEPT2022

4. TITLE AND SUBTITLE

Modulating Neural Signaling in the Treatment of Pancreatic Cancer

5a. CONTRACT NUMBER
W81XWH- 21-1-0901

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Timothy C. Wang, MD.
Susan Bates, MD.

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Columbia University Irving Medical Center
1130 St. Nicholas Ave. Rm 901
New York, NY 10032

8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Development 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

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignant diseases, with an overall survival (OS) rate of 9%1-3. The resistance of PDAC to treatment has been attributed in part to the tumor microenvironment and the complex desmoplastic stroma, which comprises numerous cells including nerves4,5. Although the least well-studied, recent evidence has suggested a role for nerves in the development of cancer. All solid tumors, apart from CNS tumors, are innervated by axonal fibers arising from the peripheral nervous system (PNS). In most solid tumors, there is a marked increase in neural density and nerve size during cancer growth6,7. Experimental model systems have shown a direct contribution of nerves to the development of prostate cancer8,9, basal cell carcinoma10, breast cancer11, and from our group, of gastric cancer12,13. We recently reported for the first time that parasympathetic signaling can profoundly suppress pancreatic cancer growth14, and this inhibitory effect of muscarinic signaling has now been confirmed in models of breast cancer11. The effect of nerves on tumor growth may be direct or indirect. Nerves modulate growth directly through interactions with cancer cells or indirectly through interaction with the tumor microenvironment (TME). In early development and during regeneration, nerves promote the growth of stromal cells derived from the mesenchymal blastema15. The purpose of this research is to investigate both the direct and indirect effects of muscarinic cholinergic signaling in suppressing PDAC.

15. SUBJECT TERMS

NONE LISTED

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

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignant diseases, with an overall survival (OS) rate of 9%[1-3]. The resistance of PDAC to treatment has been attributed in part to the tumor microenvironment and the complex desmoplastic stroma, which comprises numerous cells including nerves[4,5]. Although the least well-studied, recent evidence has suggested a role for nerves in the development of cancer. All solid tumors, apart from CNS tumors, are innervated by axonal fibers arising from the peripheral nervous system (PNS). In most solid tumors, there is a marked increase in neural density and nerve size during cancer growth[6,7]. Experimental model systems have shown a direct contribution of nerves to the development of prostate cancer[8,9], basal cell carcinoma[10], breast cancer[11], and from our group, of gastric cancer[12,13]. We recently reported for the first time that parasympathetic signaling can profoundly suppress pancreatic cancer growth[14], and this inhibitory effect of muscarinic signaling has now been confirmed in models of breast cancer[11]. The effect of nerves on tumor growth may be direct or indirect. Nerves modulate growth directly through interactions with cancer cells or indirectly through interaction with the tumor microenvironment (TME). In early development and during regeneration, nerves promote the growth of stromal cells derived from the mesenchymal blastema[15]. The *purpose* of this research is to investigate both the direct and indirect effects of muscarinic cholinergic signaling in suppressing PDAC.

KEYWORDS

Vagus nerve, Cholinergic, Muscarinic, Bethanechol, Pancreatic adenocarcinoma, cholinergic muscarinic receptor 1 (Chrm1, M1R).

ACCOMPLISHMENTS

What were the major goals of the project?

Major Goals for the 0-12-Month Reporting Period				
Major Goal	**Title**	**Timeline (months)**	**Milestone/ target date (Month)**	**Percent Complete**
Specific Aim 1: What are the mechanisms by which M1 muscarinic receptor signaling suppresses pancreatic cancer stem cells, leading to an inhibition of PDAC growth?				
Major Task 1	Define the optimal kinetics and signaling pathways mediating growth suppression by M1R *Milestone (s) Achieved: Demonstrate the effect of Kras signaling on muscarinic receptor signaling in PDAC.*	1-24	24	50%
Major Task 2	Define the mechanism of action of muscarinic suppression of PDAC cancer stem cells. *Milestone (s) Achieved: Identify and validate the downstream pathways in PDAC epithelium leading to suppression of tumor growth by muscarinic signaling.*	1-36	36	30%
Major Task 3	Examine combination of a beta-blocker (ICI) and bethanechol in the treatment of PDAC. *Milestone (s) Achieved: Determine the pharmacodynamics interaction and synergistic*	24-36	36	0%

	effect of a potential combination of beta-blockade and muscarinic stimulation.			
Specific Aim 2: Do muscarinic agonists suppress pancreatic tumorigenesis by modulating the immune microenvironment?				
Major Task 1	Determine whether loss of M1R in immune cells promotes PDAC initiation and growth. *Milestone (s) Achieved: Demonstrate the effect of M1R loss in the bone marrow on PDAC incidence, tumor growth rate and survival.*	6-36	36	20%
Major Task 2	Complete enrollment and collection of PBMCs and FFPE tissue from patients treated with bethanechol and generation of KPC mice treated with bethanechol. *Milestone (s) Achieved: Patient samples collected and banked, PBMCs and FFPE tissue from KPC tumor bearing mice harvested and banked.*	1-12	12	80%
Major Task 3	Immune profiling of PBMCs from humans and mice with PDAC treated with bethanechol. *Milestone (s) Achieved: Immune profiles of human and mouse PBMCs generated and analyzed.*	12-24	24	0%
Major Task 3	Spatial and quantitative analysis of the TIME in PDAC from humans and mice treated with bethanechol. *Milestone (s) Achieved: Demonstrate the effect of muscarinic stimulation on T cell density within the tumor stroma and distance to tumor epithelium.*	12-36	36	0%
Specific Aim 3. Is muscarinic agonism safe for patients and effective against PDAC; can it be combined with chemotherapy, and does if lead to detectable changes in tissue markers or outcome?				
Major Task 1	Protocol development and IRB approval *Milestone (s) Achieved: IRB approval and activation of study at CUIMC*	0-3	3	100%
Major Task 2	Patient enrollment *Milestone (s) Achieved: Complete enrollment of 33 patients on study.*	4-30	30	3%
Major Task 3	Analysis of study endpoints *Milestone (s) Achieved: Multifactorial analysis of efficacy of bethanechol in suppressing PDAC growth including biomarkers, kinetic analysis and surgical outcomes; submission of manuscript for peer review.*	30-36	36	0%

What was accomplished under these goals?

Specific Aim 1: What are the mechanisms by which M1 muscarinic receptor signaling suppresses pancreatic cancer stem cells, leading to an inhibition of PDAC growth?

Major Task 1: Define the optimal kinetics and signaling pathways mediating growth suppression by M1R ***(Site 1., Dr. Wang****).*

In *subtask 1* we have generated dose and time courses for cancer stem cell (CSC) suppression by muscarinic agonists. We analyzed the cell viability index of pilocarpine (broad muscarinic receptor agonist) and McN-A-343 (M1R selective muscarinic receptor agonist. Consistent with our prior data, the IC50 of pilocarpine in the Panc1 cell line was quite high (1.4 mM), but the IC50 of McN-A-343 was much lower (257 µM). As demonstrated in our previous work, suppression of cancer stem cells occurs at a lower drug dose. In Panc1 cells, the EC50 of pilocarpine in suppressing the CD44+/CD24+/EpCAM+ putative cancer stem cell population with 160 µM (**Figure 1**).

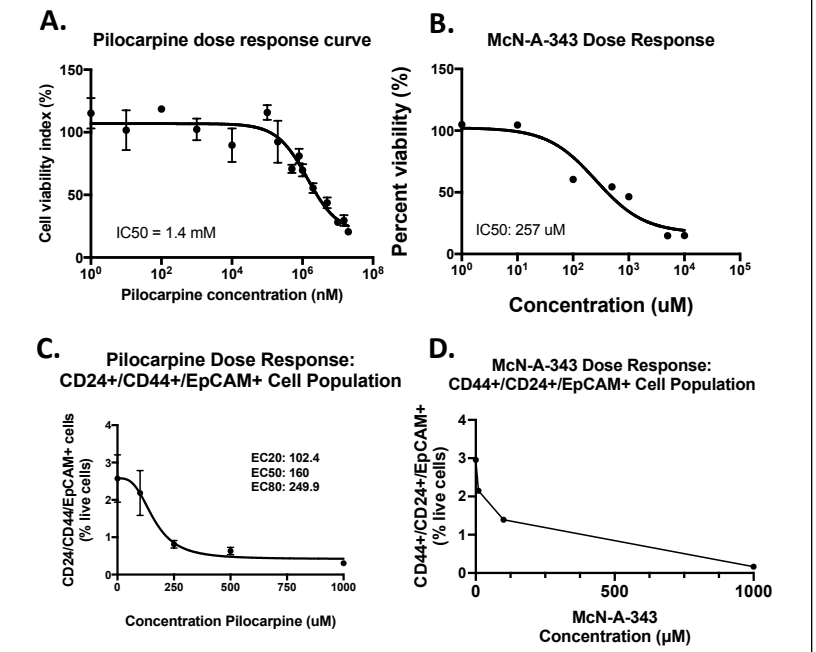

Figure 1. Muscarinic agonist dose curves. MTT assay assessing the effect of the nonselective muscarinic agonist, Pilocarpine (**A**), and M1R selective muscarinic agonist, McN-A-343 (**B**) on cell viability at different drug doses. Effect of pilocarpine (**C**) and McN-a-343 (**D**) on the CD44+/CD24+/EpCAM+ cancer stem cell population in Panc1

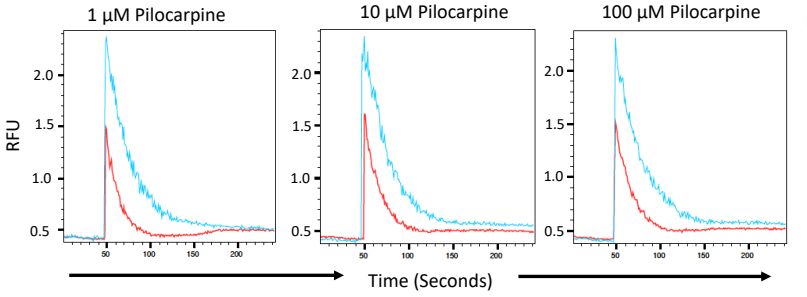

Figure 2. Pilocarpine induced Calcium flux in WT (Red) and Kras mutant (Blue) HEK293 cells. HEK293 cells were stably transduced with M1R and $Kras^{G12D}$ mutant or control Kras constructs. Calcium flux was measured using flow cytometry and the Calcium flux assay kit (Abcam). Cells were loaded with the 520AM dye and a baseline flurecence reading was obtained on the flow cytometer. At 50 seconds pilocarpine was added at either 1, 10, 0r 100 µM. The change in the relative florescence units were measured over time.

In *subtask 2* we examined the effect of Kras mutations on M1R G-protein signaling. The Human epidermal keratinocyte (HEK293) cell line was stably transduced with a KrasG12D construct in addition to ectopic expression of M1R. The effect of pilocarpine treatment on second messenger signals, calcium flux was measured using flow cytometry. Increased release of intracellular calcium was consistently seen in Kras mutant cells compared with Kras WT cells over three separate doses of pilocarpine (**Figure 2**). These findings are inconsistent with published data demonstrating that oncogenic Kras mutation suppresses intracellular secretion of Calcium from the endoplasmic reticulum[16]. This finding suggest that muscarinic signaling may disrupt the suppressive effects of Kras mutations on intracellular calcium flux and promoting differentiation of cancer stem cells and apoptosis. Further studies are ongoing examining the effect of Kras mutations on downstream ERK and PI3K activation, G-protein—beta arrestin interactions and receptor recycling.

*Major Task 2: Define the mechanism of action of muscarinic suppression of PDAC cancer stem cells (****Site 1. Dr. Wang****).*

In *Subtask 1* we aimed to determine the role of suppression of neurotrophin signaling by examining neurotrophin expression in PDAC cells. We treated the Panc1 cell line with muscarcinic agonists and

antagonists, extracted RNA and examined relative gene expression changes of a large panel of neurotrophins and neurotrophic factors compared to untreated cells. Interestingly we found that gene expression changes clustered most closely depending on drug selectivity (drugs targeting all five muscarcinic receptor vs. M1R selective drugs) rather than activity (agonist vs. antagonist). This suggests that receptor selectivity may be important factor in developing more potent targeted therapies aimed at treating PDAC. We searched the data for neurotrophin or neurotrophic factors that showed the top differentially expressed genes between cells treated with the M1R agonist (McN-A-343) and the M1R antagonist (Pirenzepine) (**Figure 3**). Two of the most up-regulated genes after pharmacological inhibition of M1R were ARTN (Artemin) and NTF4 (Neurotrophin 4). Artemin has been associated with increased invasion in pancreatic[17] and other cancers. NTF4 (neutrotrophin-4) mediates axon guidance and has recently been associated with worse outcomes in colorectal cancer and inhibition of autophagy[18].

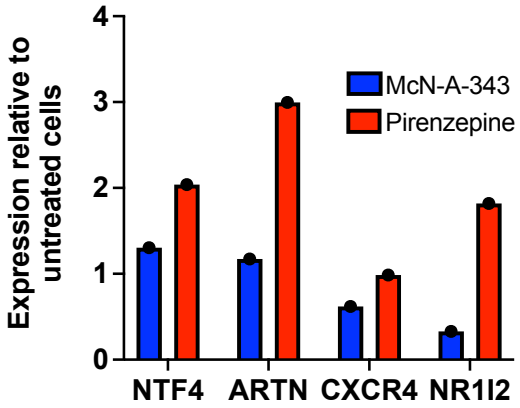

Figure 3. Top differentially expressed neurotrophins treated with M1R selective agonist and antagonists. Panc1 cells were treated with 10µM McN-A-343 (M1R selective agonist, blue bars) and Pirenzepine (M1R selective antagonist, red bars). Neurotrophin expression was measured using a neurotrophin quantitative RT-PCR array (Qiagen) and compared to expression in untreated Panc1 cells. The neurotrophins that showed the greatest difference between the M1R agonist and antagonists are shown here.

CXCR4 and NR1I2 were most suppressed after stimulation of M1R with McN-A-343. The CXCR4/CXCL12 axis has been well studied in PDAC and has been shown to play a role in the progression and development of PDAC. Suppression of CXCR4 has been shown to re-sensitize PDAC cells to gemcitabine after the development of resistance[19]. We have found that treatment with the cholinergic agonist, bethanechol, suppresses circulating levels of CXCL12 in patients treated on our phase 0 window of opportunity study (See Specific Aim 2, Major Task 2; **Figure 6C**). These findings provide further support for combining cholinergic stimulation with bethanechol in combination with gemcitabine and nab-paclitaxel in Specific Aim 3.

In *subtask 2* we aimed to generate an optimal RNAseq data set from CD44+/CD24+/EpCAM+ CSCs sorted from PANC1 cells. However, we found that the CSC markers were inconsistent between difference PDAC cell lines. For example, the MiaPaCa cell line does not express EpCAM, so the optimal markers to analyze CSCs for each PDAC cell line are unknown. These differences between cell lines would make downstream analysis and confirmation studies more challenging (*Subtask 4*). Therefore, we

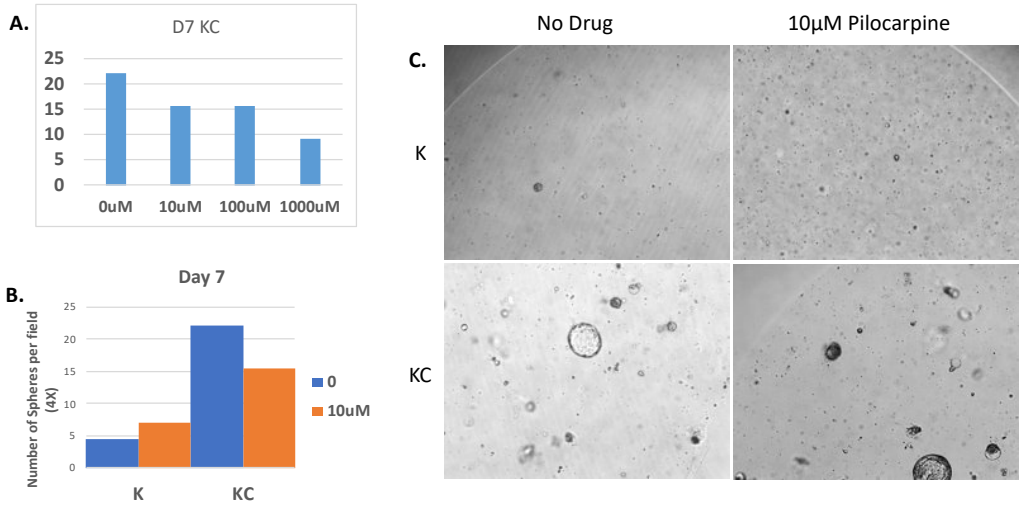

Figure 4. Suppression of pancreas organoid growth with muscarinic stimulation. A. A dose curve of pilocarpine mediated suppression of pancreatic organoid growth was generated using organoids derived from KC mice at 8 weeks of age. Average number of organoids per visual field were counted for each treatment dose. **B**. Effect of pilocarpine (orange bars) on pancreatic organoid growth in Kras WT (K) and Kras^{G12D} (KC) pancreatic organoids. **C**. Representative images of WT (K) and KrasG12D (KC) organoids treated with no drug or 10µM pilocarpine.

adjusted our strategy to focus on generating optimal RNAseq data sets from WT and Kras mutant pancreas organoids to assess the differential effects of Kras signaling on growth (Kras WT) and inhibition (Kras mutant) after treatment with cholinergic agonists. The growth of organoids in culture is a functional readout of CSCs and therefore will give us a more physiologic representation of the effects of cholinergic stimulation on stem-like properties in PDAC. We have developed an optimal dose curve of pilocarpine treatment in the organoids in Specific Aim 1, Major task 1 (**Figure 4**) and have prepared RNA from these samples in preparation for RNAseq analysis. Confirmatory studies of candidate differentially expressed genes will remain the same as proposed (*Subtask 4*).

Major Task 3: Examine combination of a beta-blocker (ICI) and bethanechol in the treatment of PDAC ***(Site 1 and 2, Drs Wang and Bates)****.*
No data to report (planned for 12-36 month period).

Specific Aim 2: Do muscarinic agonists suppress pancreatic tumorigenesis by modulating the immune microenvironment?
*Major Task 1: Determine whether loss of M1R in immune cells promotes PDAC initiation and growth (****Site 1., Dr. Wang****).*
Subtask1: Impact of Chrm1-/- bone marrow on initiation and progression of PDAC—KC mice with Chrm1-/- bone marrowy transplantation. We have initiated the bone marrow transplant studies, however we found that due to the mixed background of our KC and KPC line we have had to utilize littermate donor mice on the same background (KC;Chrm1$^{-/-}$ and KPC;Chrm1$^{-/-}$). Generating such mouse lines requires a large number of mice. To reduce the number of mice needed for these studies, we are obtaining KPC mice that have been backcrossed on to a C57Bl6 background consistent with the Chrm1$^{-/-}$ line to allow the two syngeneic lines to be maintained independently.

Subtask 2: Orthotopic transplantation in to M1R knockout recipient mice. We have established an orthotopic PDAC transplantation model and begun assessing the effect of M1R loss (Chrm1-/-) in the tumor microenvironment on PDAC growth kinetics. In this model one million syngeneic Panc02 PDAC cancer cells expressing a luciferase reporter are injected into the pancreas of either control WT recipients or Chrm1$^{-/-}$ recipient mice. Tumor size and luciferase activity are measured to assess tumor growth. Preliminary analysis has shown that there is a trend towards increased tumor volumes in in Chrm1$^{-/-}$ mice (Figure 5). We are currently evaluating a larger cohort of mice to determine if these preliminary effects are significant.

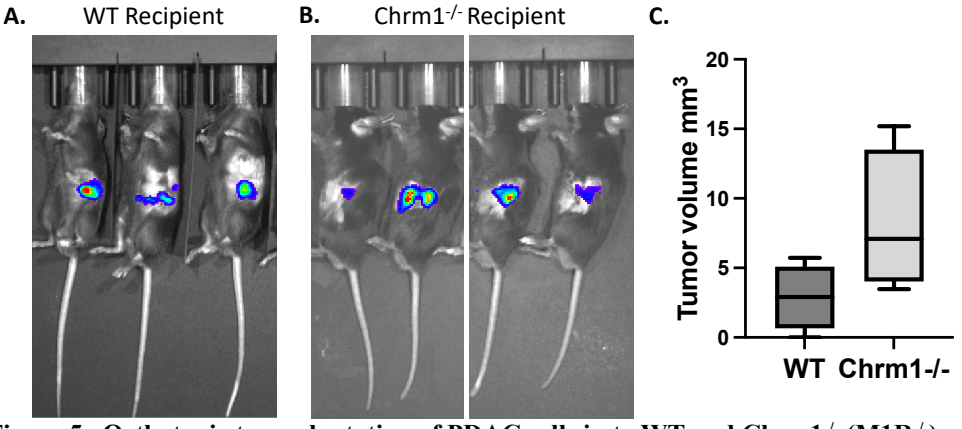

Figure 5. Orthotopic transplantation of PDAC cells in to WT and Chrm1$^{-/-}$ (M1R$^{-/-}$) recipient mice. One million murine Panc02 cells expressing a luciferace construct were orthotopically transplanted into the pancreas of WT or Chrm1-/- recipient mice. **A**. Representative bioluminescent images of Panc02 tumors in WT mice one week after transplantation. **B**. Representative bioluminescent images of Panc02 tumors in Chrm1-/- mice one week after transplantation. **C.** measurement of tumor volume in WT (n=3) and Chrm1-/- (n=3) recipient mice three weeks after transplantation.

Major Task 2: Complete enrollment and collection of PBMCs and FFPE tissue from patients treated with bethanechol and generation of KPC mice treated with bethanechol ***(Site 1 and 2, Drs Wang and Bates)****.*
Subtask 1: To date we have enrolled at total of 12 out of 15 evaluable patients on the phase 0, window of opportunity study of bethanechol in patients with resectable PDAC. Demographics of the enrolled patients closely aligns with published patient characteristics for resectable disease (**Figure 6A**). The median age of

enrolled patients was 74 (range 59-85) and with more male (58%) than female (42%) subjects. The R0 resection rate was 64%. The drug is very well tolerated with only grade 1 and 2 adverse events recorded and all adverse events were expected effects seen with cholinergic stimulation such as hot flashes, urinary frequency and increased saliva (**Figure 6 B**). Preliminary analysis of inflammatory cytokines shows that after bethanechol treatment there is a trend towards suppression of inflammatory markers, with some exceptions (**Figure 6 C, D**). For example, CCL5 is increased after bethanechol treatment in the majority of patients (**Figure 6 C**). This chemokine plays a role in the recruitment of T lymphocytes into the tumor immune environment. Other notable changes demonstrated include suppression of CXCL12 (**Figure 6C**) and suppression of TNFα (**Figure 6D**). Staining for tissue biomarkers shows a trend towards suppression of Ki-67 and CD44 in patients treated with bethanechol compared to untreated control subjects (**Figure 6E, F**).

Subtask 2: We have generated 12 control mice and 8 bethanechol treated KPC mice and banked PBMCs and FFPE for analysis in Major tasks 2 and 3 for Specific Aim 2.

A.

Age -- Yr	
Median	74
Range	59-85
Sex -- no. (%)	
Female	5 (42)
Male	7(58)
Pancreatic Tumor location -- no. (%)	
Head	10 (83)
Body/tail	2 (17)
Days on treatment	
Median	8
Range	7-13
Status of surgical margins --- no. (%)	
R0	7 (64)
R1	4 (36)
Tumor Stage -- no. (%)	
IB	3 (37)
IIA	2 (18)
IIB	4 (36)
III	2 (18)
IV	0 (0)
Lymphovascular invasion --no. (%)	6 (54)
Perineural invasion -- no. (%)	8 (72)

B.

Event	Number (%)	G1	G2
Cholinergic			
Hot flashes	4 (33)	2	2
Urinary frequency	2 (16)	2	
Hyperhydrosis	1 (8)	1	
Increased saliva	2 (16)	2	
Nausea	1 (8)	1	

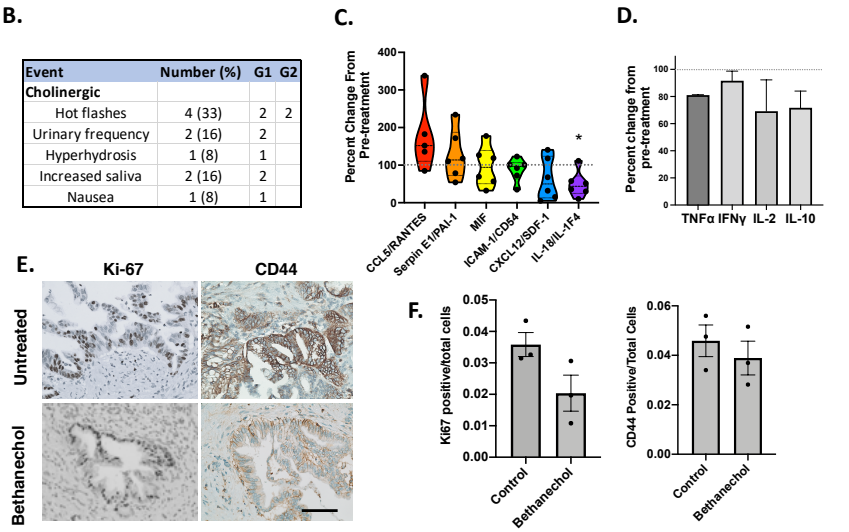

Figure 6. Preliminary data from the phase 0, window of opportunity study of bethanechol in PDAC patients prior to surgery. **A**. Patient characteristics (note- pathology pending on patient #12 at the time of this report). **B**. Adverse events. **C**. Plasma cytokine and chemokine expression in paired patient samples before and after treatment with bethanechol. Plasma cytokines were analyzed on a dot blot array. Pixel density was analyzed using ImageJ and data are presented as the percent change in cytokine expression from pre-treatment levels. **D**. Analysis of cytokines with lower plasma expression using mesoscale technology. Data is reported as the percent change from pre-treatment levels for three paired patient samples. **E**. Representative immunohistochemistry of tissue biomarkers, Ki-67 and CD44 in resected PDAC from patients treated with bethanechol compared to stage and grade matched historical controls. **F.** Quantitation of biomarker staining from the first three patients enrolled on the study compared to stage and grade matched historical controls.

Major Task 3: Spatial and quantitative analysis of the TIME in PDAC from humans and mice treated with bethanechol ***(Site 1 and 2, Drs Wang and Bates)****.*
Nothing to report (planned for 24-36 month period).

Specific Aim 3. Is muscarinic agonism safe for patients and effective against PDAC; can it be combined with chemotherapy, and does if lead to detectable changes in tissue markers or outcome?
*Major Task 1: Protocol development and IRB approval (****Site 2. Dr. Bates****).*
Timeline of protocol development:
IND exemption letter on 5/24/2021 from the FDA.
Protocol was approved first by our GI CUIMC Disease Based Team
Approved by the Protocol Review and Monitoring Committee on 7/29/2021
Approved by the Institutional Review Board on 9/11/2021
Approved by the DOD on 12/7/2021

Approved by the Fiscal Support Committee on 1/25/2022
Site initiation visit on 2/3/22.
Milestone Achieved: Study officially open on 2/3/22
First patient enrolled: 7/17/2022

*Major Task 2: Patient enrollment (**Site 2. Dr. Bates**)*
At this time, we have enrolled one patient with two potential subjects currently undergoing eligibility screening. We continue to actively screen all new pancreatic cancer patients seen in the Pancreas Center for eligibility in a clinical trial at Columbia including this Phase II neoadjuvant trial. Trial enrollment is assessed weekly during the Gastrointestinal Oncology Disease Management Team research meetings. Promotion of ongoing open trials occurs at weekly Pancreas Cancer Multidisciplinary Conferences. We discuss in the sections below, details of our strategies to identify patients for the study.

We have generated additional data to support the feasibility of one of our clinical trial endpoints – the growth rate kinetics. Our manuscript describing the overall method and application of the kinetic equations to data from 3,033 patients with metastatic or locally advanced PDAC has been accepted for publication in *The Oncologist* **(1)** We are very confident that the equations apply equally well to the pancreatic primary site as to metastatic disease. In addition, we have re-analyzed data from our 45-patient neoadjuvant trial with gemcitabine, docetaxel, and capecitabine (GTX) that formed the basis for our endpoint calculations (2). We have shown in this that we are able to determine both growth (***g***) and regression (***d***) rates in these patients, and that demonstration of growth, rather than regression alone, predicts a poor outcome. This may be an obvious finding but has never been demonstrated mathematically.

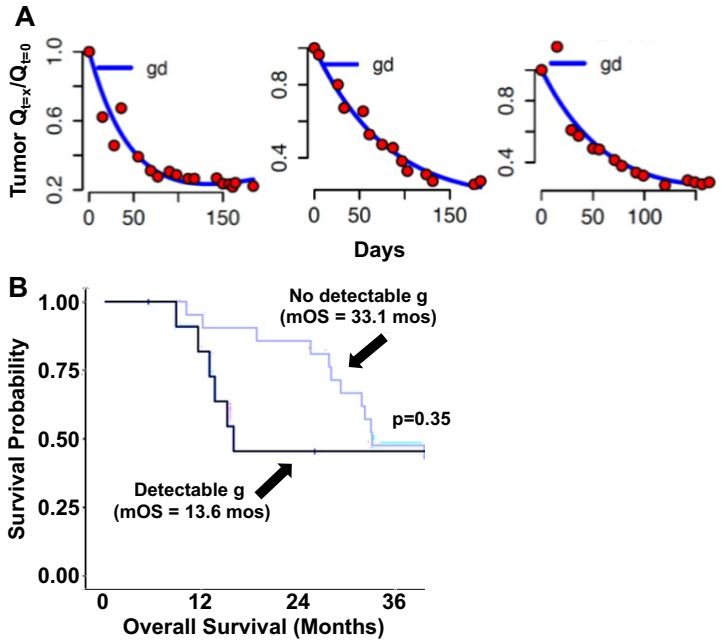

Figure 3
A. Examples of individual patient data fit to the growth/regression equation.
B. Kaplan-Meier plots of OS based on whether a rate of tumor growth was detected or not detected during neoadjuvant therapy.

Detection of a Rate of Tumor Growth to Inform the Efficacy of Neoadjuvant Therapy. To assess the feasibility of using this approach in the neoadjuvant setting, we applied our mathematical model of tumor growth and regression to data from a prospective study of 45 patients treated with GTX in the neoadjuvant setting (2). Examples of individual patient CA19-9 values fit to the growth rate equation are shown in **Figure 7A**. The red dots represent actual CA19-9 values, which are declining throughout the treatment period. Superficially there is no way to tell that these datapoints represent anything except successful treatment. However, the three examples in **A** fit the ***gd*** growth rate equation, showing that our model is detecting a growing treatment-resistant tumor fraction and estimate its rate, designated ***g***. Although recurrence date is not known, Kaplan-Meier analysis of OS found a trend toward improved survival for patients in whom no detectable tumor growth was detectable during neoadjuvant GTX compared to those in whom a detectable ***g*** emerged during therapy (**Figure 7B**). These analyses suggest that our method is robust enough to detect differences between the pre and post-bethanechol periods in patients on the study.

*Major Task 3: Analysis of study endpoints (**Sites 1 and 2, Drs. Bates and Wang**).*
Nothing to report (planned for 30-36 month period).

What opportunities for training and professional development has the project provided?
While this project was not intended to provide training and professional development opportunities, the project has provided training opportunities to the co-investigator, Dr. White and post-doctoral fellow, Dr. Feijing Wu. Dr. White has participated in all aspects of the project and gained experience in clinical trial design, study activation and conduct and correlative analysis. She has had the opportunity to present this work as an oral abstract at the AACR Special Conference on Pancreatic Cancer. Dr. Feijing Wu has gained experience in working with pancreatic cancer organoid models. Dr.White is currently drafting the manuscript from the Phase 0 study, which as noted above appears to have generated some data supporting proof of concept.

How were the results disseminated to communities of interest?
Preliminary clinical trial results and pre-clinical data was presented as an oral abstract to the AACR Special Conference on Pancreatic Cancer in September 2022. The results have also been shared by oral presentation to the Columbia University Pancreas Center to educate the clinical teams about the ongoing clinical trials and the data supporting these trials. Additionally, Dr Susan Bates has published in The Oncologist (*in press*).

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

Goals for 12-24-month reporting period			
Aims/Tasks/Milestones	**Timeline** (Months)	**Site 1** Wang	**Site 2** Bates
Specific Aim 1: What are the mechanisms by which M1 muscarinic receptor signaling suppresses pancreatic cancer stem cells, leading to an inhibition of PDAC growth?			
Major Task 1: Define the optimal kinetics and signaling pathways mediating growth suppression by M1R	1-24	X	
Subtask2: Examine the effect of Kras mutation on M1R G-protein signaling. • Examine the effect of G-protein inhibitors on downstream ERK and PI3K activation in Kras WT and mutant cells • Examine the effect of Kras on G-protein beta-arresting interactions and receptor recycling. • Investigate if mutant Kras contributes to the inhibitory effect of M1R signaling by analysis of M1R signaling in mouse and human organoids.	12-24	X	
Major Task 2: Define the mechanism of action of muscarinic suppression of PDAC cancer stem cells.	1-36	X	X
Subtask 2: Generate an optimal RNAseq data set from WT and Kras mutant organoids treated with muscarinic agonists. • Subtask 2b: RNAseq on treated WT and Kras mutant organoids; preliminary pathways analysis on genes with greatest difference in expression.	12-24	X	
Subtask 3. Bioinformatics analysis to investigate mechanism of action of cholinergic agonists on Kras mutant organoids	12-24	X	
Specific Aim 2: Do muscarinic agonists suppress pancreatic tumorigenesis by modulating the immune environment?			
Major Task 1: Determine whether loss of M1R in immune cells promotes PDAC initiation and growth	3-36		
Subtask 1: Impact of Chrm1-/- bone marrow on initiation and progression of PDAC.	12-30	X	

• Bone marrow transplant of Chrm1-/- cells into KC recipient mice. Anticipate transplantation of initial 10 out of 28 planned mice.			
Subtask 2. Impact of Chrm1-/- bone marrow on survival • Bone marrow transplantation of Chrm1-/- cells into KPC recipient mice. • Orthotopic transplantation in to M1R recipient mice.	12-36	X	
Major task 3: Immune profiling of PBMCs from humans and mice with bethanechol treated PDAC.	12-24		
Subtask 1. High parametric immune profiling (CyTOF) of human and mouse PBMCs	12-24	X	
Subtask 2. Quantitative multiplex immunofluorescence of the T cell subpopulations in the TIME.	12-36	X	X
Specific Aim 3: Is muscarinic agonism safe for patients and effective against PDAC; can it be combined with chemotherapy, and does it lead to detectable changes in tissue markers or outcome?			
Major Task 2: Patient enrollment	4-30		
Subtask 1. Recruitment of patients from CUIMC Pancreas Center. • Evaluation of eligibility, consent, central review process, enrollment on study.	12-30		X

IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Nothing additional to report for now.

What was the impact on other disciplines?
Nothing additional to report for now.

What was the impact on technology transfer?
Nothing to report at present.

What was the impact on society beyond science and technology?
Nothing additional to report at present.

CHANGES/PROBLEMS

Changes in approach and reasons for change
In ***Specific Aim 1, Major task 2, subtask 2*** we aimed to generate an optimal RNAseq data set from CD44+/CD24+/EpCAM+ CSCs sorted from PANC1 cells. However, we found that the CSC markers were inconsistent between difference PDAC cell lines. For example, the MiaPaCa cell line does not express EpCAM, so the optimal markers to analyze CSCs for each PDAC cell line are unknown. These differences between cell lines would make downstream analysis and confirmation studies more challenging (*Subtask 4*). Therefore, we adjusted our strategy to focus on generating optimal RNAseq data sets from WT and Kras mutant pancreas organoids to assess the differential effects of Kras signaling on growth (Kras WT) and inhibition (Kras mutant) after treatment with cholinergic agonists. The growth of organoids in culture is a functional readout of CSCs and therefore will give us a more physiologic representation of the effects of cholinergic stimulation on stem-like properties in PDAC. This adjustment is minor and not expected to change the overall scope of the research aim significantly.

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

Specific aim 2, Major task 1: Impact of Chrm1$^{-/-}$ bone marrow on initiation, growth kinetics and survival of KC and KPC mice.

We have found that the background of our KC and KPC mouse strain housed in our lab is not on a pure C57BL6 background. The Chrm1$^{-/-}$ line is on a C57BL6 background and therefore incompatible for Chrm1$^{-/-}$ bone marrow transplantation into the KC and KPC cell lines. To resolve this, we are obtaining a KPC line on a C57BL6 background from Dr. Iok Christine Chio at our institution. This will allow us to complete the experiments proposed in Specific aim 2, Major task 1 without any major changes to experimental design but has slightly delayed the initiation of the bone marrow transplant experiments. We anticipate that we will still be able to complete the proposed experiments in the timeframe outlined in the SOW.

Specific aim 3, Major task 2: Patient enrollment.

Our DOD-funded Phase II study in the neoadjuvant setting administers gemcitabine and nab-paclitaxel in the neoadjuvant – the first 2 months being the two agents alone and the second two months combining with bethanechol. We appreciate the DOD recognition of the novelty of this concept, and the striking preclinical work underlying the addition of bethanechol to drive cholinergic stimulation to decrease pancreatic cancer growth. As we noted in the 3rd quarter report, enrollment to the study has been a greater challenge than expected. While we are a high volume Pancreas Center, with 4 active Pancreas surgeons, the COVID pandemic again impacted our program in the winter, in Jan-Feb 2022, with operating rooms closing and some surgeries being delayed and therefore referred to other locations. We also had a subsequent period of six- eight weeks with only a single patient with borderline resectable disease- suggesting that perhaps pandemic related delays had resulted in some patient upstaging. During the second quarter only one patient was identified with borderline resectable disease. That patient had Parkinson's disease and so was not eligible. A month ago, our team prescreened one patient with borderline resectable disease with a strong family history of breast and pancreatic cancer who likely had a homologous recombination germline mutation and therefore needed to be treated with a platinum agent. During the 3rd quarter we screened two patients with borderline resectable disease this week – one from Staten Island who could not commute; the other enrolled and has now received 3 cycles of chemotherapy per protocol. He received 2 cycles of gemcitabine-nab-paclitaxel alone; and then bethanechol was initiated as per protocol. He has received 2 of the next two cycles. His scan showed a 20% reduction in tumor size. We will repeat imaging at the end of cycle 4 when the plan is for him to undergo a Whipple resection. We have just screened 2 more patients with apparent borderline resectable disease -one had metastatic disease and the other became medically quite ill before enrollment.

While we are steadily seeking patients, this is a much lower rate of accrual than we had expected. As mentioned above, Dr. White and Dr. Bates have engaged in the education of our surgeons regarding neoadjuvant therapy and our study rationale and eligibility. We have weekly multidisciplinary Pancreas cancer meetings in which each patient is separately reviewed, and we seek those with borderline resectable disease as potential candidates. During these meetings, we have discussed the remarkable demonstration by Dr. Wang in murine models that cutting the vagus nerve leads to more advanced pre-cancerous lesions and a greater incidence of invasive carcinomas. We have again discussed with the surgeons the preclinical data showing that bethanechol can reverse these effects and prolong survival in the animal models. Dr. Bates discussed in detail with the surgeons the lack of data supporting a bias that the stronger chemotherapy in the FOLFIRINOX regimen will be more likely to render surgical success. Indeed a completely new registry study, termed PURPLE, recently published from Australia (3) again confirmed that FOLFIRINOX and gemcitabine/nab-paclitaxel are not statistically different in the treatment of metastatic disease and by extrapolation support the observations in the Southwest Oncology Group SWOG 1505 study showing that the gemcitabine and nab-paclitaxel regimen is either the same as or perhaps somewhat better than FOLFIRINOX **(4)**. These data also mirror our own findings in a database study from the Veterans Administration, examining tumor growth rates and survival in Veterans who received gemcitabine or FOLFIRINOX (5).

We have been engaged in multiple discussions with our team regarding the best strategy to improve accrual, beyond increasing the awareness of the surgeons. At the 3rd quarter report, I discussed a strategy to amend the study to add cisplatin to the combination. Adding cisplatin to gemcitabine and nab-paclitaxel has been studied in a Southwest Oncology Group trial that just completed with the regimen upfront in biliary cancers so that a safe dose of the regimen is well-established. There has been no comparison study of gemcitabine + nab-paclitaxel vs.

gemcitabine + nab-paclitaxel + cisplatin in the neoadjuvant setting, in pancreatic cancer, but we feel that the combination is at least equivalent, based on growth rate kinetics we have performed using data obtained from Daniel D. Von Hoff, who is the physician in chief and director of translational research at Translational Genomics Research Institute, and current Virginia G. Piper Distinguished Chair for Innovative Cancer Research at HonorHealth Clinical Research Institute. Dr. Von Hoff was the senior author on the original publication demonstrating activity of the three-drug gemcitabine + nab-paclitaxel + cisplatin combination in pancreatic cancer (6).

While that strategy is still under discussion, we have also recently recognized that some patients do not benefit from the addition of cisplatin, which will compromise the dose of gemcitabine and nab-paclitaxel, as well as causing significant cumulative bone marrow toxicity. As described above there are numerous data sets that support the notion that FOLFIRINOX and gemcitabine/nab-paclitaxel are equivalent in the real world. Cisplatin is clearly important for patients with cancers bearing BRCA2 mutations, but this is not the case in the majority of patients.

Our third strategy remains to seek one or more outside partners – and we have already reached out to Dr. Paul Oberstein who wrote the initial bethanechol Phase 0 study before departing Columbia for New York University Medical Center. Dr. Oberstein is interested in the Phase II study; and the study is under discussion at his institution.

Changes that had significant impact on expenditures
Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Nothing to report.

Significant changes in use of care of human subjects
Nothing to report.

Significant changes in use or care of vertebrate animals.
Nothing to report.

Significant changes in use of biohazards and/or select agents.
Nothing to report.

PRODUCTS

Publications, conference papers, and presentations

Journal Publications:

Yeh C, Zhou M, Sigel K, Jameson G, White R, Safyan R, Saenger Y, Hecht E, Chabot J, Schreibman S, Juznya B, Ychou M, Conroy T, Fojo T, Manji GA, Von Hoff D, Bates SE**.** Tumor growth rate informs treatment efficacy in metastatic pancreatic adenocarcinoma: application of a growth and regression model to pivotal trial and real-world data. The Oncologist. 2022; *in press*.

Books or other non-periodical, one-time publications: Nothing to report

Other publications, conference papers and presentations:

Cholinergic modulation of T lymphocytes in pancreatic adenocarcinoma. White, R.A., Waterbury, Q.T., Ochiai, Y., Zamechek, L.B., Bates, S.E., Wang, T.C. American Association for Cancer Research Special Conference: Pancreatic Cancer, Boston MA, September 13-16, 2022. Oral presentation selected from proffered abstracts.

Websites or other internet sites
Nothing to report.

Technologies of techniques
Nothing to report.

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals contributing to project during reporting period	
Name	Timothy C. Wang, MD.
Project Role	Initiating PI
Researcher Identifier (ORCID)	0000-0001-5730-3019
Nearest person month worked	0.9 (as approved in DoD Grant)
Contribution to project	Experimental Design of all the activities in the Wang Lab as outlined in the DoD approved Statement of Work (SOW), Oversight, DoD and Research compliance, basic research component of the DoD approved W81XWH-21-1-0901 Project.
Funding Support	W81XWH-21-1-0901
Name	Susan Bates, MD.
Project Role	Collaborating/Partnering PI
Researcher Identifier (ORCID)	0000-0001-6708-0330
Nearest person month worked	0.9 (as approved in DoD Grant)
Contribution to project	Experimental Design of all the activities in the Bates Lab and Clinic; including screening and recruiting patients, obtaining consent, assessing eligibility, office visits of participants, managing chemotherapy, interacting with surgeons, and managing adverse events, and managing tasks as outlined in the DoD approved Statement of Work (SOW), Oversight, DoD and Research compliance, clinical component of the W81XWH-21-1-0901 Project.
Funding Support	W81XWH-21-1-0902
Name	Ruth White, MD., PhD.
Project Role	Co-investigator
Researcher Identifier (ORCID)	0000-0002-2108-8932
Nearest person month worked	1.2 + 0.24 (as approved in DoD Grant)

Contribution to project	Work with both Initiating PI (Dr Wang) and Partnering PI (Dr Bates) using her expertise in lab and clinical studies to conduct experiments, document and analyze results; as well as handle the clinical aspects of the DoD approved Statement of Work (SOW).
Funding Support	W81XWH-21-1-0901, W81XWH-21-1-0902
Name	Helen Remotti, MD.
Project Role	
Researcher Identifier (ORCID)	0000-0003-1555-9299
Nearest person month worked	0.18 (as approved in DoD Grant)
Contribution to project	Diagnosing, staging and grading tumors, characterization of morphologic and molecular alterations in tumors; supervision of final scoring of the biomarker endpoints and the R0 status following surgery.
Funding Support	W81XWH-21-1-0901, W81XWH-21-1-0902
Name	J. Labella
Project Role	Research Technician
Researcher Identifier (ORCID)	N/A
Nearest person month worked	1.8 (as approved in DoD Grant)
Contribution to project	Work with the team and help with technical aspects of the research, including mouse husbandry and animal studies.
Funding Support	W81XWH-21-1-0901
Name	Feijing Wu, MD.
Project Role	Post-doctoral Fellow
Researcher Identifier (ORCID)	0000-0001-9198-7960
Nearest person month worked	6.0 (as approved in DoD Grant)
Contribution to project	Work with the PIs and team on the DoD approved Statement of Work (SOW), to conduct experiments, document and analyze results.
Funding Support	W81XWH-21-1-0901

Changes in the active other support of the PD/PIs or senior/key personnel since the last reporting period:

The Initiating PI, Dr Timothy Wang, MD, alongwith a collaborator, Dr Jianwen Que, MD, PhD, got an NIH grant to study GI junction stem cells as the origin of Barrett's esophagus and GI cancer. The NIH grant is not overlapping scientifically or otherwise with the DoD grant. The DoD grant is focused on examining the effect of muscarinic agonists in suppressing pancreatic tumors by modulating microenvironment, mechanisms of suppressing pancreatic cancer stem cells and testing bethanechol in a Phase IIA clinical trial to query borderline resectable tumors.
The Partnering PI, Dr Susan Bates, MD, got an extension of her Pancreatic Cancer Action Network grant on animal studies to find a safe dose for romidepsin and MZ735. This is not overlapping scientifically or otherwise with the DoD grant. The DoD grant is focused on examining the effect of muscarinic agonists in suppressing

pancreatic tumors by modulating microenvironment, mechanisms of suppressing pancreatic cancer stem cells and testing bethanechol in a Phase IIA clinical trial to query borderline resectable tumors.

Other organizations involved as partners
Nothing to report at this time.

SPECIAL REPORTING REQUIREMENTS:
N/A at present

APPENDICES:
Accepted Manuscript attached. Please note it is a proof version, the sticky note has information regarding the acknowledgment of our Wang-Bates DoD grant.

REFERENCES

1 Hidalgo, M. Pancreatic cancer. *New England Journal of Medicine* **362**, 1605-1617 (2010).
2 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2018. *CA Cancer J Clin* **68**, 7-30, doi:10.3322/caac.21442 (2018).
3 Olive, K. P. *et al.* Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* **324**, 1457-1461, doi:10.1126/science.1171362 (2009).
4 Demir, I. E., Friess, H. & Ceyhan, G. O. Neural plasticity in pancreatitis and pancreatic cancer. . *Nat Rev Gastroenterol Hepatol* **12**, 649-659 (2015).
5 Sun XJ, Jiang TH, Zhang XP & AW, M. Role of the tumor microenvironment in pancreatic adenocarcinoma. *Front Biosci (Landmark Ed)* **21**, 31-41 (2016).
6 Ayala GE, D. H., Powell M, Li R, Ding Y, Wheeler TM, Shine D, Kadmon D, Thompson T, Miles BJ, Ittmann MM, Rowley D. Cancer-related axonogenesis and neurogenesis in prostate cancer. *Clin Cancer Res* **14**, 7593-7603 (2008).
7 Albo D *et al.* Neurogenesis in colorectal cancer is a marker of aggressive tumor behavior and poor outcomes. *Cancer* **117**, 4834-4845 (2011).
8 Magnon, C. *et al.* Autonomic nerve development contributes to prostate cancer progression. *Science* **341**, 1236361, doi:10.1126/science.1236361 (2013).
9 Zahalka, A. H. *et al.* Adrenergic nerves activate an angio-metabolic switch in prostate cancer. *Science*, In press (2017).
10 Peterson, S. C. *et al.* Basal cell carcinoma preferentially arises from stem cells within hair follicle and mechanosensory niches. *Cell Stem Cell* **16**, 400-412, doi:10.1016/j.stem.2015.02.006 (2015).
11 Kamiya, A. *et al.* Genetic manipulation of autonomic nerve fiber innervation and activity and its effect on breast cancer progression. *Nat Neurosci* **22**, 1289-1305, doi:10.1038/s41593-019-0430-3 (2019).
12 Hayakawa, Y. *et al.* Nerve growth factor promotes gastric tumorigenesis through aberrant cholinergic signaling. *Cancer cell* **31**, 21-34 (2017).
13 Zhao, C. M. *et al.* Denervation suppresses gastric tumorigenesis. *Sci Transl Med* **6**, 250ra115, doi:10.1126/scitranslmed.3009569 (2014).
14 Renz, B. W. *et al.* Cholinergic Signaling via Muscarinic Receptors Directly and Indirectly Suppresses Pancreatic Tumorigenesis and Cancer Stemness. *Cancer Discov* **8**, 1458-1473, doi:10.1158/2159-8290.CD-18-0046 (2018).
15 Boilly, B., Faulkner, S., Jobling, P. & Hondermarck, H. Nerve Dependence: From Regeneration to Cancer. *Cancer Cell* **31**, 342-354, doi:10.1016/j.ccell.2017.02.005 (2017).
16 Pierro, C., Cook, S. J., Foets, T. C., Bootman, M. D. & Roderick, H. L. Oncogenic K-Ras suppresses IP(3)-dependent Ca(2)(+) release through remodelling of the isoform composition of IP(3)Rs and ER luminal Ca(2)(+) levels in colorectal cancer cell lines. *J Cell Sci* **127**, 1607-1619, doi:10.1242/jcs.141408 (2014).
17 Ceyhan, G. O. *et al.* The neurotrophic factor artemin promotes pancreatic cancer invasion. *Annals of surgery* **244**, 274-281, doi:10.1097/01.sla.0000217642.68697.55 (2006).

18 Yang, Z. *et al.* Upregulated NTF4 in colorectal cancer promotes tumor development via regulating autophagy. *Int J Oncol* **56**, 1442-1454, doi:10.3892/ijo.2020.5027 (2020).
19 Arora, S. *et al.* An undesired effect of chemotherapy: gemcitabine promotes pancreatic cancer cell invasiveness through reactive oxygen species-dependent, nuclear factor kappaB- and hypoxia-inducible factor 1alpha-mediated up-regulation of CXCR4. *J Biol Chem* **288**, 21197-21207, doi:10.1074/jbc.M113.484576 (2013).

REFERENCES for S.E. Bates (Partnering PI) section

1. Yeh C, Zhou M, Sigel K, Jameson G, White R, Safyan R, Saenger Y, Hecht E, Chabot J, Schreibman S, Juznya B, Ychou M, Conroy T, Fojo T, Manji GA, Von Hoff D, Bates SE. Tumor growth rate informs treatment efficacy in metastatic pancreatic adenocarcinoma: application of a growth and regression model to pivotal trial and real-world data. The Oncologist. 2022; in press.
2. Sherman WH, Chu K, Chabot J, Allendorf J, Schrope BA, Hecht E, et al. Neoadjuvant gemcitabine, docetaxel, and capecitabine followed by gemcitabine and capecitabine/radiation therapy and surgery in locally advanced, unresectable pancreatic adenocarcinoma. Cancer. 2015;121(5):673-80.
3. Santucci J, Tacey M, Thomson B, Michael M, Wong R, Shapiro J, Jennens R, Clarke K, Pattison S, Burge M, Zielinski R, Nikfarjam M, Ananda S, Lipton L, Gibbs P, Lee B. Impact of first-line FOLFIRINOX versus Gemcitabine/Nab-Paclitaxel chemotherapy on survival in advanced pancreatic cancer: Evidence from the prospective international multicentre PURPLE pancreatic cancer registry. Eur J Cancer. 2022 Oct;174:102-112. doi: 10.1016/j.ejca.2022.06.042. Epub 2022 Aug 18. PMID: 35988408.
4. Lo W, Zureikat A. Neoadjuvant therapy in pancreatic cancer: a review and update on recent trials. Curr Opin Gastroenterol. 2022 Sep 1;38(5):521-531. doi: 10.1097/MOG.0000000000000874. Epub 2022 Jul 15. PMID: 35881969.
5. Sigel K, Zhou M, Sigel C, Park YA, Mutetwa T, Nadkarni G, Yeh C, Polak P, Fojo T, Wisnivesky JP, Bates SE. Gemcitabine plus nab-paclitaxel versus FOLFIRINOX for unresected pancreatic cancer: Comparative effectiveness and evaluation of tumor growth in Veterans. Semin Oncol. 2021 Feb;48(1):69-75. PMID 33714591.
6. Jameson GS, Borazanci E, Babiker HM, Poplin E, Niewiarowska AA, Gordon MS, et al. Response Rate Following Albumin-Bound Paclitaxel Plus Gemcitabine Plus Cisplatin Treatment Among Patients With Advanced Pancreatic Cancer: A Phase 1b/2 Pilot Clinical Trial. JAMA Oncol. 2019;6(1):125-32.