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Clinical Trial Results

A phase 0, window of opportunity study of parasympathetic stimulation with bethanechol in localized pancreatic adenocarcinoma prior to surgery

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Abstract

Background: The parasympathetic branch of the autonomic nervous system has shown tumor-suppressive effects in preclinical models of pancreatic adenocarcinoma (PDAC) by inhibiting cancer stem cells and suppressing inflammatory cytokine production. Based on these findings, we hypothesized that bethanechol, an FDA-approved parasympathomimetic agent targeting muscarinic receptors, could enhance treatment efficacy in PDAC.

Methods: We conducted a phase 0/window of opportunity study evaluating short-term parasympathetic activation with fixed dose bethanechol (100 mg twice daily) in subjects with resectable or borderline resectable PDAC prior to surgery. The primary endpoint was a change in cell proliferation by Ki-67 expression compared to stage-matched controls. Secondary endpoints included tissue expression of stem cell markers (CD44), infiltrating immune cells (CD8a, Granzyme B, and CD68), and changes in circulating inflammatory cytokine concentrations.

Results: Seventeen patients were enrolled with 13 eligible for analysis of endpoints. Median age was 74 (59-86), 6 female (46%), all ECOG 0-1, and median duration of treatment was 8 days (7-13). R0 resections were achieved in 9 patients (69%). There was no difference in Ki67 and CD44 tissue biomarkers between bethanechol-treated and control samples. Decreased numbers of Granzyme B-expressing cells were seen in bethanechol-treated tissues. Bethanechol treatment was associated with the suppression of circulating IL-18. The most common treatment-related adverse events (TRAE) were hot flashes (30.7%), urinary frequency (15.4%), increased salivation (15.4%), hyperhidrosis (7.7%), and nausea (7.7%). There were no Grade 3 or higher adverse effects. No surgical complications were attributed to bethanechol treatment.

Conclusion: Bethanechol 100 mg twice daily is well tolerated in patients with PDAC in this small phase 0/window of opportunity study (NCT03572283). Bethanechol treatment was associated with decreased Granzyme B positive cells and decreased circulating IL-18 consistent with an anti-inflammatory role for parasympathetic muscarinic signaling in PDAC.

Key words: bethanechol; parasympathetic agonists; cholinergic agonists; muscarinic agonists; pancreatic adenocarcinoma; nerve-cancer interactions.

Lessons learned

- Stimulation of cholinergic, muscarinic receptors with the broad muscarinic receptor agonist, bethanechol, is safe and well tolerated in patients with PDAC prior to surgery.
- Bethanechol treatment results in suppression of inflammatory cytokines and Granzyme B expressing cells consistent with pharmacological activation of the cholinergic anti-inflammatory pathway.
- This study demonstrates the feasibility of repurposing FDA-approved agents to target nerve-cancer interactions to treat cancer.

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Trial Information	
Disease	Resectable or borderline resectable pancreatic adenocarcinoma
Stage of disease/ treatment	I-III
Prior therapy	No prior therapy
Type of study	Phase 0/Window of opportunity
Primary endpoints	Decrease in proliferative index by Ki67 staining.
Secondary endpoints	Surgical outcomes, tolerability, tissue biomarkers of cancer stem cells, and immune profiling.

Additional details of endpoints or study design
The primary endpoint of this study is a change in cell proliferation by Ki-67 expression in bethanechol-treated patients compared to historical stage-matched controls. A previous study utilizing the MIB-1 monoclonal antibody on paraffin-embedded tissue of resected pancreatic cancer specimens reported a mean MIB-1 labeling index of 28.1 with a standard deviation of 14.7 and staining in 94% of specimens[1] while another study reported median Ki-67 staining in 26% of pancreatic cancer nuclei.[2] We hypothesized that short term treatment with bethanechol would decrease cell proliferation and reduce Ki-67 staining based on our preclinical data.[3] With a goal of 15 analyzable pairs and assuming baseline Ki-67 expression of 28%, we would have 80% power to detect a change in Ki-67 of 12% (0.8 standard deviations) to 16% in the post-treatment specimens. The probability of a Type I error for testing the null hypothesis that the Ki-67 response difference is zero is 0.05.
The secondary endpoints were tolerability of short course bethanechol and impact on surgical complications as well as stem cell (CD44), immune cell (CD8a, Granzyme B, CD68) tissue biomarkers, and concentrations of circulating inflammatory cytokines.

Drug Information	
Generic/working name	Bethanechol
Company name	Generic
Drug type	Parasympathomimetic
Drug class	Muscarinic agonist
Dose	100 mg
Route	PO
Schedule of administration	Twice daily for 7-14 days prior to surgery.
Dose modification	Dose level—1: 50 mg twice daily offered to patients with unacceptable treatment related toxicity.

Patient characteristics

The study enrolled patients with a histologically or cytologically confirmed diagnosis of PDAC considered resectable or borderline resectable as determined by the treating surgeon. Eligible patients were ≥ 18 years of age, had an ECOG performance status of 0-1, and had no prior chemotherapy or radiation treatment. Major exclusion criteria included the use of acetylcholinesterase inhibitors or beta blockers, active peptic ulcer disease, gastrointestinal or bladder surgery within 12 months of study enrollment, and active asthma requiring daily use of rescue inhalers.

Patient Characteristics	
Sex—*N* (%)	
Female	6 (46)
Male	7 (54)
Age years—median (range)	74 (59-86)
Ethnicity—*N* (%)	
White	6 (46)
Hispanic	4 (31)
Asian	2 (15)
Black	1 (8)
Pathologic Stage—*N* (%)	
IB	3 (23)
IIA	2 (15)
IIB	4 (31)
III	4 (31)
IV	0 (0)
ECOG—*N* (%)	
0	9
1	4

Primary assessment method

The primary assessment for this study was a change in cell proliferation by Ki-67 staining in pancreatic cancer samples in bethanechol-treated patients compared to samples from stage-matched control patients (Figure 1A, B). We screened 17 patients for this study, and 16 were enrolled. Three patients withdrew consent, two after starting treatment and one prior to starting treatment. There were 13 patients evaluable for toxicity and primary and secondary endpoints, except for plasma cytokine analysis. For plasma cytokines, there were only 6 available paired samples for analysis (Figure 2). Surgical outcomes are reported in Table 1.

Primary Assessment Method	
Title	Biomarker of tumor cell proliferation
Number of patients screened	17
Number of patients enrolled	16
Number of patients evaluable for toxicity	13
Number of patients evaluated for efficacy	13
Evaluation method	Ki67 immunohistochemistry

Outcome Notes: Response assessment staining and quantification: See Figure 1a and b for representative IHC staining and quantification respectively. Tissue staining quantification was performed using the HALO Quantitative Image Analysis platform. No significant difference was detected in Ki67 staining between bethanechol-treated and control samples.

Secondary Assessment Method—Tissue Biomarkers	
Title	Tissue cancer stem cell and immune biomarkers
Number of patients evaluated for efficacy	13
Evaluation method	CD44, CD68, CD8, Granzyme B immunohistochemistry

Outcome notes: Response assessment staining and quantification: See Figure 1 C-J for representative IHC staining and quantification. Tissue staining quantification was performed using the HALO Quantitative Image Analysis platform. Granzyme B positive cells were markedly reduced in treated samples. Other immune biomarkers were unchanged.

Secondary Assessment Method—Circulating Inflammatory Cytokines	
Title	Tissue cancer stem cell and immune biomarkers
Number of patients evaluated for efficacy	6
evaluation method	Plasma inflammatory cytokine concentrations

Outcome notes: Circulating inflammatory cytokines were measured in patients before and after bethanechol treatment. Plasma cytokines concentration was measured using Luminex inflammatory cytokine arrays (Figure 2). Only IL-18 and GRO-α (CXCL1) were significantly altered, with IL-18 reduced while GRO-α was increased.

Serious adverse events

There were no serious adverse events (SAES).

General toxicity profile			
Adverse event	Number (%)	Grade 1 (%)	Grade 2 (%)
Hot Flashes	4 (30)	2 (15.4)	2 (15.4)
Urinary Frequency	2 (15.4)	2 (15.4)	
Hyperhydrosis	1 (8)	1 (8)	
Increased Saliva	2 (15.4)	2 (15.4)	
Nausea	1 (8)	1 (8)	

Discussion

Pancreatic adenocarcinoma (PDAC) is a highly aggressive malignancy with a five-year overall survival rate of approximately 10%.[4] Progress in developing effective therapies has been slow, largely due to the immunosuppressive tumor microenvironment and dense fibrotic stroma. Among patients eligible for surgical resection at diagnosis, more than 80% will experience recurrence, with a median time to recurrence of 6.9 months without further systemic therapy.[5] For patients with advanced or metastatic disease, optimal chemotherapy extends median overall survival to just 12 months.[6,7] These challenges underscore the urgent need for more effective treatment strategies targeting PDAC.

The peripheral nervous system plays an essential role in tumor dynamics, including in PDAC. The pancreas is innervated by sensory, sympathetic, and parasympathetic fibers. Sensory fibers are found in the thoracic splanchnic and vagus nerves, while sympathetic fibers reside in the splanchnic nerves and parasympathetic fibers in the vagus nerve.[8] Early denervation of sensory nerves through capsaicin administration in autochthonous pancreatic cancer models during the neonatal period has been shown to impair tumor development and improve survival.[9] These effects can be seen as early as the premalignant pancreatic intraepithelial neoplasia (PanIN) stages.[9]

The sympathetic and parasympathetic branches of the autonomic nervous system have opposite effects on PDAC initiation and growth. Chronic restraint stress in murine models activates the sympathetic nervous system resulting in catecholamine release, which promotes PDAC growth by activating B2 adrenergic receptors.[10] In contrast, autonomic parasympathetic signaling suppresses PDAC growth. The vagus nerve provides parasympathetic innervation to the pancreas, playing a key role in excitatory stimulation that influences both exocrine and endocrine secretion.[8] In autochthonous animal models, subdiaphragmatic vagotomy accelerates PDAC growth and emergence of premalignant PanIN lesions. Vagal parasympathetic stimulation inhibits PDAC growth due, at least in part, to the suppression of CD44-expressing cancer stem cells and a reduction in the proliferative capacity of the tumor epithelium.[3,11] This occurs primarily through signaling via the cholinergic muscarinic receptor 1 (Chrm1). These effects are mimicked by activation of muscarinic receptors with the parasympathomimetic agent, bethanechol.[3] These preclinical findings provide the rationale for this Phase 0 window of opportunity study evaluating bethanechol in patients with PDAC amenable to surgical resection.

In this study, patients with PDAC who were deemed eligible for upfront surgery were enrolled and treated with bethanechol 100 mg twice daily for at least 7 days. Plasma was collected before the start of treatment and at the time of surgery. Formalin-fixed paraffin embedded tissue from the resected tumor was obtained for tissue biomarker analysis and historical stage-matched tissue was used as control. Surgical outcomes and complications were evaluated.

Seventeen patients were screened and 16 were eligible for enrollment. Three patients withdrew consent either shortly after starting treatment or before treatment. Of the 13 evaluable patients, the median age was 74 (59-86) and 6 (46%) were female. The majority of the tumors (85%) were located in the head of the pancreas, and the remaining 15% were located in the body or tail of the pancreas. The median duration of treatment was 8 days (7-13), which is in line with time to surgery at our institution. Nine patients (69%) achieved an R0 resection consistent with published surgical outcomes.[12] Treatment-related adverse events (TRAE) were generally mild and as expected due to the cholinergic effects of bethanechol. The most common TRAE was hot flashes, which was seen in 30% of the patients. There were no grade 3 or higher TRAEs. Few complications were seen in patients following surgery and none were attributed to bethanechol treatment. The most common was gastroparesis, which was observed in 2 patients (15%) and is a common complication of Whipple surgery, occurring in up to 61% of patients.[13] Together, these data demonstrate the safety and tolerability of bethanechol in PDAC patients.

The study did not meet the primary endpoint of decreased Ki67 staining in patients with bethanechol treatment. Similarly, we found no difference in expression of the PDAC stem cell marker CD44 between bethanechol-treated and control tissue. This may be the result of a relatively short course

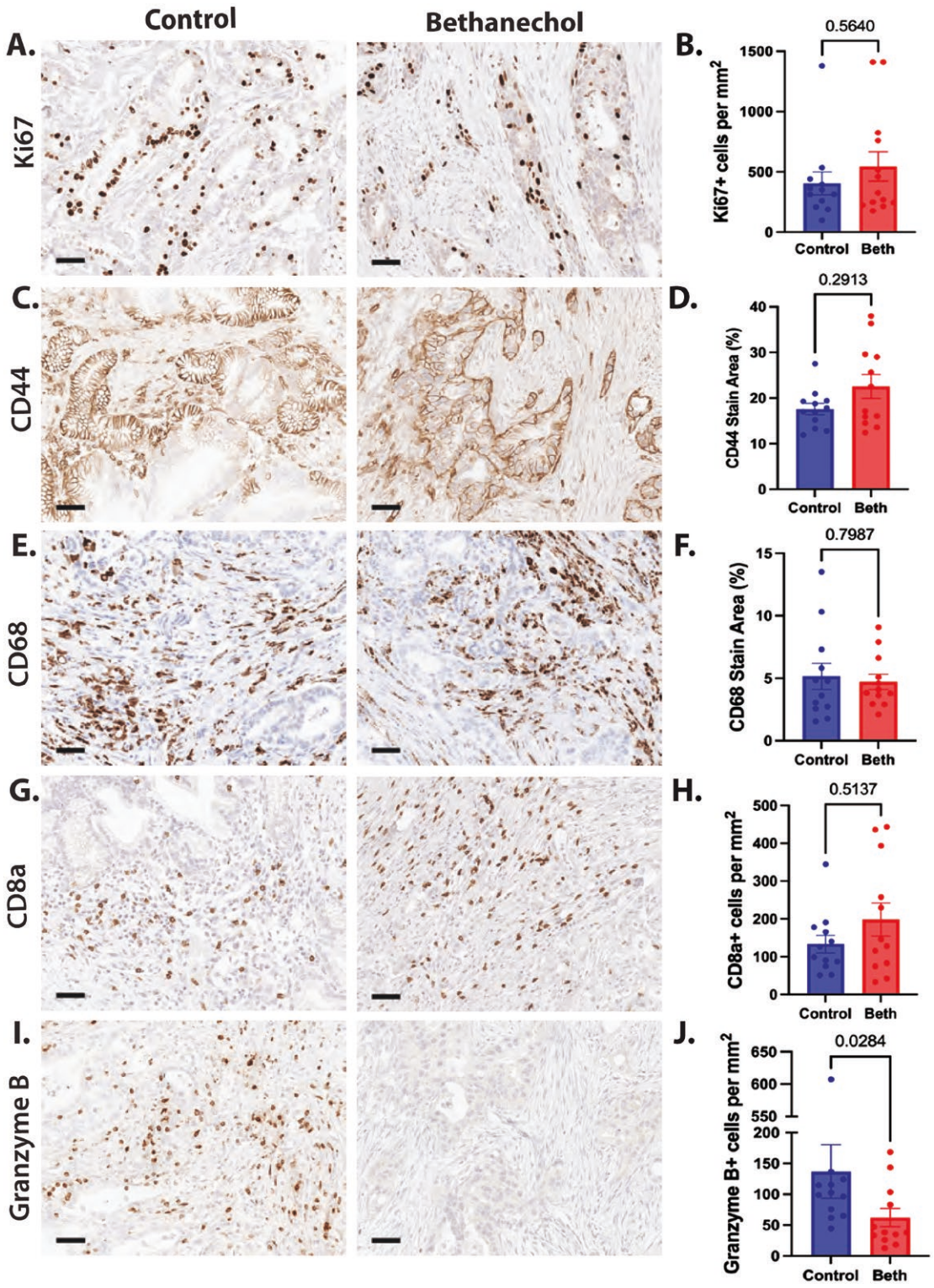


Figure 1. Tissue biomarker expression in resected PDAC from control (left panels) or bethanechol-treated (right panels) patients. Representative examples are shown in the panels: A. Ki67 immunohistochemistry (IHC) in control and bethanechol-treated samples. B. Average Ki67 positive cells per mm^2 tissue area. C. Representative CD44 IHC staining in control and bethanechol-treated samples. D. Average CD44 staining area in non-stromal tissue. E. Representative CD68 macrophage marker staining in samples from control and bethanechol-treated patients. F. Average CD68 stain area. G. Representative CD8a staining in control and bethanechol-treated patients. H. Average number of CD8a positive cells per mm^2 tissue area. I. representative Granzyme B staining in control and bethanechol-treated patients. J. Average number of Granzyme B positive cells per mm^2 tissue area. Tissue quantification was performed using the HALO Quantitative Image Analysis platform. Results are expressed as the mean +/- SEM from control (n = 12) or bethanechol treated (n = 13) patient PDAC surgical resection specimens, Mann Whitney test p values shown on graphs. IHC scale bars, 50 μm.

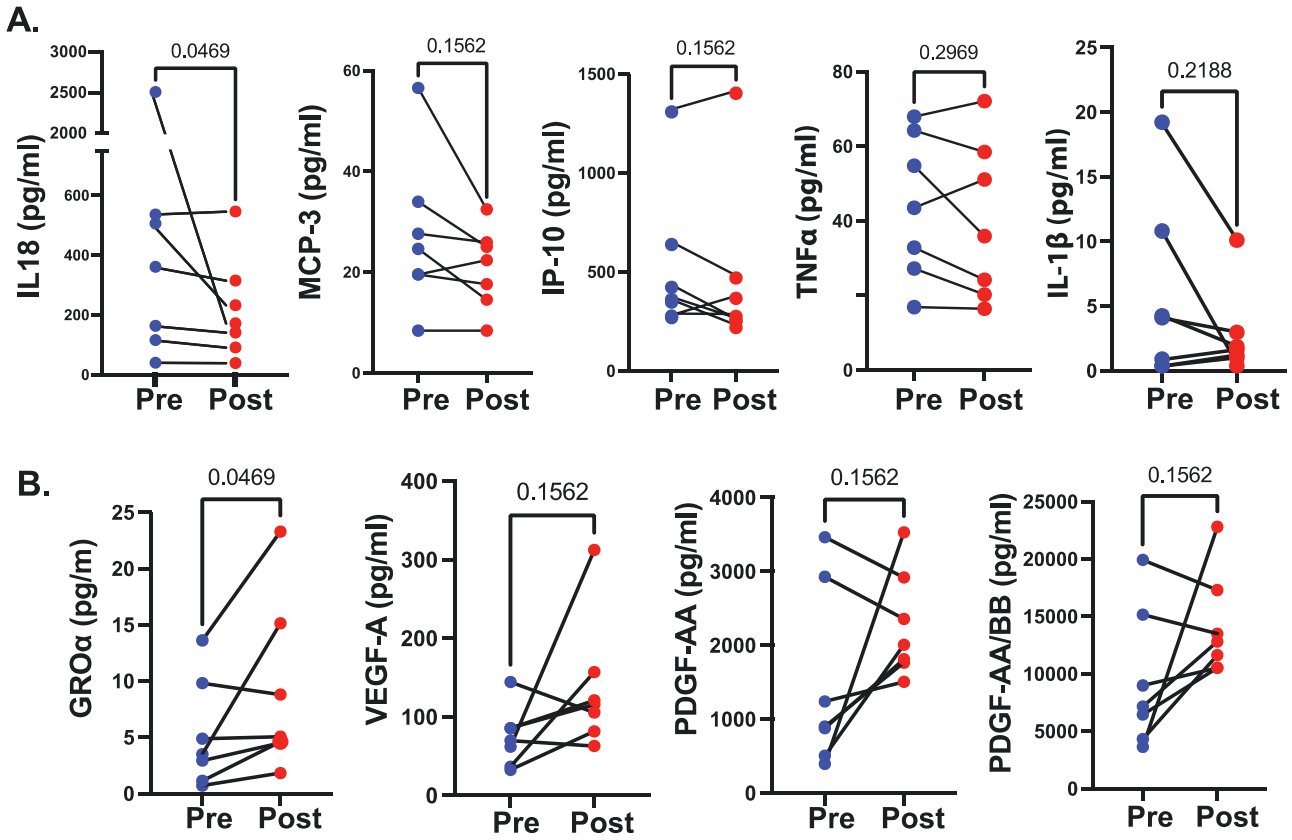


Figure 2. Effect of bethanechol treatment on circulating inflammatory cytokines. A. cytokines with most significant decrease following bethanechol treatment. B. Cytokines with the most significant increase following bethanechol treatment. Cytokine concentration was measured using Luminex inflammatory cytokine assays. Paired pre- and post- treatment cytokine concentrations for each patient were analyzed. Significance was assessed using Wilcoxon matched-pairs signed rank tests and *P* values shown on graphs.

Table 1. Pathology and surgical outcomes.

PATHOLOGY AND SURGICAL OUTCOMES	
Days on treatment—Median (Range)	8 (7-13)
Tumor location—*N* (%)	
Pancreatic head	11 (85)
Pancreatic body or tail	2 (15)
Status of surgical margins—*N* (%)	
R0	9 (69)
R1	4 (31)
Pathologic Stage—*N* (%)	
IB	3 (23)
IIA	2 (15)
IIB	4 (31)
III	4 (31)
IV	0 (0)
Lymphovascular Invasion—*N* (%)	7 (54)
Perineural Invasion—*N* (%)	9 (70)
Post-operative complications—*N* (%)	
Gastroparesis	2 (15)
Ileus	1 (8)
Acute Kidney Injury	1 (8)
Pulmonary Edema	1 (8)
Chyle Leak	1 (8)

of treatment and was likely limited by the inability to obtain paired biopsy tissue before and after treatment to directly assess the effect of bethanechol in this small patient population. However, we did see alterations in immune parameters.

Beyond its role in pancreatic endocrine and exocrine function, the vagus nerve is known to regulate systemic inflammation. This cholinergic anti-inflammatory pathway helps counteract inflammation caused by bacterial infections and sepsis.[14] Vagal efferent nerves detect cellular damage and inflammation in the periphery, transmitting these signals centrally. Afferent vagal pathways mediate the release of catecholamines from splenic ganglion nerves, stimulating acetylcholine secretion from lymphocytes. In the spleen, acetylcholine binds to nicotinic cholinergic receptors on splenic macrophages, suppressing the release of inflammatory cytokines such as TNF-α.[15] Consistent with this mechanism, our study observed increased TNF-α levels in PDAC-bearing mice following vagotomy.

Our preclinical studies demonstrated that vagotomy increased tissue macrophages, while bethanechol treatment suppressed them.[3] However, we found no difference in tumor macrophages in the bethanechol-treated patients compared to controls. There was also no difference in CD8a positive T lymphocytes. Surprisingly, we found significantly fewer Granzyme B positive cells in the bethanechol-treated patients compared to controls, which raises the question of whether muscarinic signaling may suppress lymphocyte activation. To further assess the immune effects of bethanechol in PDAC,

we compared the expression of cytokines from paired plasma samples taken from patients before and after bethanechol treatment and found a significant reduction in circulating IL-18 levels following treatment (Figure 2A). IL-18 is a pro-inflammatory cytokine secreted by various cell types, including macrophages, dendritic cells, and epithelial cells, and regulates several critical downstream pathways, including those involved in cell survival and proliferation.[16] Elevated levels of circulating IL-18 have been correlated with poor prognosis in patients with PDAC,[16] and there is evidence that IL-18 contributes to the exhaustion of T cells. This is mediated by activation of the IL2/STAT and AKT/mTOR signaling pathways in lymphocytes driving upregulation of markers of T cell exhaustion such as PD-1, TIM-3, and LAG3.[17] In addition to IL-18, we also saw a trend toward suppression of a number of other inflammatory cytokines after bethanechol, including MCP-3, IP-10, TNFα, and IL-1β, all of which have been associated with worse outcomes in PDAC (Figure 2A).[16,18-23] The global anti-inflammatory effects of bethanechol may help counter chronic inflammation, a key driver of PDAC initiation and progression, thereby restoring an effective anti-tumor immune response.

Interestingly, the cytokines most elevated after bethanechol treatment are associated with angiogenesis (VEGF) or are secreted from endothelial cells (GRO-α, PDGF) (Figure 2B). There is evidence that muscarinic signaling can stimulate neovascularization and induce VEGF in tumor associated macrophages.[24] These contrasting inflammatory findings underscore the importance of further research to explore the immune effects of muscarinic signaling in PDAC.

In conclusion, this window of opportunity study of cholinergic simulation with the muscarinic agonist, bethanechol, in patients with resectable PDAC demonstrated that this treatment is safe and tolerable for patients. Tissue biomarker endpoints were not met, however exploratory inflammatory analysis demonstrated suppression of granzyme B expressing cells and suppression of inflammatory cytokines including IL-18. Providing evidence that parasympathetic anti-inflammatory activity can be recapitulated by treatment with muscarinic agonists such as bethanechol. Further mechanistic studies of muscarinic signaling in immune cell regulation and inflammation are warranted to better understand this mechanism and role in cancer initiation and progression.

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Conception, design; acquisition, analysis, and/or interpretation of data: Ruth A. White, Paul Oberstein, Timothy C. Wang, Susan E. Bates. Provided study material or patients: Ruth A. White, Beth Schrope, Michael Kluger, Kazuki Sugahara, John Chabot, Gulam Manji, Susan E. Bates. Collected and/or assembled data: Ruth A. White, Qiongyu Shi, Nadine Kuriakose. Data analysis and interpretation: Ruth A. White, Valeria Mezzano-Robinson, Helen Remotti, Susan E. Bates. Writing: Ruth A. White, Qiongyu Shi, Nadine Kuriakose, Susan E. Bates. Final approval: Ruth A. White, Valeria Mezzano-Robinson, Qiongyu Shi, Nadine Kuriakose, Beth Schrope, Michael Kluger, Kazuki Sugahara, John Chabot, Gulam Manji, Paul Oberstein, Helen Remotti, Timothy C. Wang, Susan E. Bates.

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Conflicts of Interest

The authors declare no competing interest.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

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