

AWARD NUMBER: W81XWH-19-1-0683

TITLE: Optimizing a Novel Intraductal Delivery of Calcineurin Inhibitors as a Radiocontrast Infusion Formulation to Prevent Post-ERCP Pancreatitis

PRINCIPAL INVESTIGATOR: Sohail Husain, MD

CONTRACTING ORGANIZATION: Stanford University, Stanford, CA

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14. ABSTRACT An endoscopic retrograde cholangiopancreatography (ERCP) procedure is a common and life-saving gastrointestinal procedure that is performed in about half a million American each year, among which about 3-15% of patients were found to develop post-ERCP pancreatitis (PEP), the most common adverse effect of ERCP without effective preventative modalities. The current project funded by Award W81XWH-19-1-0683 was proposed based on our recent discovery showing the critical role of calcineurin (Cn) signaling pathway in the development of PEP. Its overarching goal is to optimize the delivery of calcineurin inhibitors (CnIs) to prevent PEP. In the first project years, we conducted mouse experiments designed for Aim 1, including endocrine and systemic safety testing of novel formulations of CnIs. We also developed the conceptual model to evaluate the preventive effect of rectal delivery of CnI tacrolimus (Tac) against post-ERCP pancreatitis. Our proposed project has been progressing smoothly. Our request of a second one-year non-cost extension was approved which allows us to schedule experiments to the fifth project year. In the fourth year, we have finished (1) 90% of Aim 1 to perform the safety testing of infusion with CnI formulations, (2) 90% of Aim 2 to analyze the pharmacokinetic features of rectal suppository delivery of Tac versus intravenous or intragastric administration, and (3) confirmed the preventive efficacy of rectal suppository administration of Tac in mouse models of post-ERCP pancreatitis and cerulein-induced pancreatitis. Our results demonstrate that (1) Tac or Cyclosporin A (CsA) formulation are safe at high single doses in a mouse model of post-ERCP pancreatitis, (2) rectal suppository delivery of Tac retains a higher blood Tac level compared with intravenous or intragastric administration, and (3) rectal Tac administration significantly decreases pancreatic injury and inflammation in both mouse models of pancreatitis.		

Taken together, these data suggest that rectal suppository delivery of Cn inhibitor is a promising strategy in the prophylaxis of pancreatitis including post-ERCP pancreatitis.

In the next project year, we plan to implement the rest of the experiments as proposed in the “Summary and timeline for the proposed work”. In summary, our preclinical IND-enabling studies can determine the most effective formulations and dosing, as well as the optimal routes for CnI delivery in the prevention and/or treatment against pancreatic disorders including post-ERCP pancreatitis.

15. SUBJECT TERMS

None listed.

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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Post-ERCP pancreatitis (PEP) remains a significant iatrogenic challenge. Employing mouse models, we identified that calcineurin (Cn) signaling plays a critical role in the development of PEP, suggesting that Cn inhibitors (CnIs, including tacrolimus (Tac) and cyclosporin A [CsA]) can be used in the prophylaxis of PEP. Given the unmet clinical needs for effective preventative modalities against PEP, our study aims to develop novel CnI formulations and the optimal routes of administration to prevent PEP. Specifically, we will (1) evaluate the safety profile of CnI formulations in the setting of ERCP models, (2) further explore the optimal delivery (e.g., via the rectum) and CnI formulations that may include non-steroidal anti-inflammatory drugs (NSAIDs). This preclinical IND-enabling research project will suggest the most effective CnI formulations and the optimal routes of administration in the prevention of post-ERCP pancreatitis.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Calcineurin, calcineurin inhibitor, ERCP, pancreatitis, post-ERCP pancreatitis, tacrolimus, cyclosporin A, endocrine toxicity, systemic toxicity, rectal suppository

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

An endoscopic retrograde cholangiopancreatography (ERCP) procedure is a common and life-saving gastrointestinal procedure that is performed in about half a million American each year, among which about 3-15% of patients were found to develop post-ERCP pancreatitis (PEP), the most common adverse effect of ERCP without effective preventative modalities.

The current project funded by Award W81XWH-19-1-0683 was proposed based on our recent discovery showing the critical role of calcineurin (Cn) signaling pathway in the development of PEP. Its overarching **goal** is to optimize the delivery of calcineurin inhibitors (CnIs) to prevent PEP. The Aims are:

Aim 1. Determine the safety profile of the novel Cn inhibitor-RC formulation via a rectal route.

Aim 2. Evaluate the efficacy of the rectal Cn inhibitor-RC formulations.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

We have performed the following during this reporting period (the fourth project year):

Aim 1. We have confirmed that administration of the Cn inhibitor formulations in mice are safe and do not specifically cause endocrine or systemic toxicity (90% completed during project years 1-4)

Aim 2. We have performed efficacy and pharmacokinetic testing using the rectal administration of the Cn inhibitor formulations in mice. Our results demonstrate that rectal Tac was effective in reducing pancreatic injury and inflammation in both PEP and cerulein models. Pharmacokinetic studies revealed that the rectal administration of Tac helped achieve optimal blood levels of Tac over an extended time compared to intravenous or intragastric delivery. (90% completed during project years 1-4).

Regulatory work. We have had a pre-IND meeting and are now on the last leg of further regulatory development and chemistry manufacturing and controls (CMC). (80% finished).

Please see Appendix A for details.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

This project was not intended to provide training or professional development opportunities.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The results from our study were disseminated to communities of interest through publications and presentations. During the reporting period, we have published two manuscripts:

- Rectal administration of tacrolimus protects against post-ERCP pancreatitis in mice. *Pancreatology*. 2023 Sep 24;S1424-3903(23)01715-5. doi: 10.1016/j.pan.2023.09.080. PMID: 37778935
- Preclinical safety evaluation of calcineurin inhibitors delivered through an intraductal route to prevent post-ERCP pancreatitis demonstrates endocrine and systemic safety. *Pancreatology*. 2023 Jun;23(4):333-340. doi: 10.1016/j.pan.2023.03.009. PMID: 37031049

We also presented our results as posters at the 2023 Stanford Bio-X Interdisciplinary Initiatives Seed Grants Symposium and Poster Session.

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

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

During the next reporting period, we will confirm that the levels of tacrolimus in the serum as well as in the pancreas are below the non-toxic range. The pharmacokinetics will also complete the remaining 10% of aim 2, which is to perform efficacy testing of the rectal administration of the calcineurin inhibitor formulations in preventing post ERCP pancreatitis. We have determined in several studies, including from what we have published, that a threshold level of tacrolimus in the serum is necessary for efficacy. The pharmacokinetics from the blood as well as now additionally from the pancreas will confirm that there is the minimal amount of tacrolimus available and for what periods of time with rectal administration. The next year of the no-cost extension will also allow us to complete the remaining 20% of the IND filing to the FDA. We have had a pre-IND meeting and are now on the last leg of further regulatory development and chemistry manufacturing and controls (CMC).

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have established that calcineurin inhibitor formulations are safe (without endocrine and systemic toxicities) and effective (with the ability to significantly decrease pancreatic injury and inflammation in mouse models of post-ERCP pancreatitis [PEP]). We also defined that rectal suppository delivery can retain a higher level of blood tacrolimus compared with intragastric or intravenous administration, in addition to its efficacy in the prophylaxis of pancreatitis including PEP. Our findings significantly support the next step IND filing and the ensuing clinical trials. Products that may result from this research could provide an efficacious and safe preventative for pancreatitis including PEP.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*

- *adoption of new practices.*

Upon the completion of this preclinical project, we will be able to put together a strategic plan with multiple partners move our novel calcineurin inhibitor formulations from concept to initial FDA approval, necessary clinical trials, final FDA approval, and finally to the commercial market.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Nothing to Report

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

- The relocation of our lab from University of Pittsburgh, Pittsburgh, PA to Stanford University, Palo Alto, CA in 2019 as well as the relocation from Stanford University main campus to Stanford Research Park significantly delayed and interfered with the implementation of the proposed work.
- COVID-19 pandemic-related policy and safety measures also slowed down our research activities.
- However, barring more obstacles, we believe we should be able to complete the rest of the proposed work in the fifth project year.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or 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

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

• **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

- Yu-Chu Lin, Jianbo Ni, Gayathri Swaminathan, Asna Khalid, Monique T Barakat, Adam R Frymoyer, Cheng-Yu Tsai, Ying Ding, Judy-April Murayi, Thottala Jayaraman, Ronald Poropatich, Rita Bottino, Li Wen, Georgios I Papachristou, Sunil G Sheth, Mang Yu, Sohail Z Husain. Rectal administration of tacrolimus protects against post-ERCP pancreatitis in mice. *Pancreatology*. 2023 Sep 24:S1424-3903(23)01715-5. Acknowledgement: Yes
- Jianbo Ni, Asna Khalid, Yu-Chu Lin, Monique T Barakat, Jing Wang, Cheng-Yu Tsai, Pasha Reza Shams Azar, Ying Ding, Judy-April Murayi, Thottala Jayaraman, Ronald Poropatich, Rita Bottino, Li Wen, Georgios I Papachristou, Gayathri Swaminathan, Mang Yu, Sohail Z Husain. Preclinical safety evaluation of calcineurin inhibitors delivered through an intraductal route to prevent post-ERCP pancreatitis demonstrates endocrine and systemic safety. *Pancreatology*. 2023 Jun;23(4):333-340. Acknowledgement: Yes

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of*

publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

We presented our results as posters at the 2023 Stanford Bio-X Interdisciplinary Initiatives Seed Grants Symposium and Poster Session.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

The Husain Lab website (www.husainlab.org) gives an overview of our research work and our accomplishments. The home page has a video for a poster presentation that disseminates the some of the work we have accomplished on this project. We actively post our research activities, publications, and accolades on the Husain Lab (@SohailHusainLab), Division of Gastroenterology, Hepatology, and Nutrition (@stanfordPedsGI), Department of Pediatrics (@stanfordPeds), and Stanford Medicine (@StanfordMed) on social media X.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

We filed the following utility patent:
For: Compositions and Methods for Preventing Post-ERCP Pancreatitis
Incentor: Sohail Z. Husain, Monique T. Barakat
U.S. PCT Patent Application No. PCT/US2021/023144
Date Filed: March 19, 2021
Stanford Ref.: S20-149
KTS Ref.: 079445-1234339-005810PC
Declaration with the World Intellectual Property Organization: July 16, 2021

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- data or databases;
- physical collections;
- audio or video products;
- software;
- models;
- educational aids or curricula;
- instruments or equipment;
- research material (e.g., Germplasm; cell lines, DNA probes, animal models);
- clinical interventions;
- new business creation; and
- other.

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: Mary Smith

Project Role: Graduate Student

Researcher Identifier (e.g. ORCID ID): 1234567

Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

1. Sohail Husain, MD

Project Role: Principal Investigator

Nearest person month worked: 12

Contribution to Project: Dr. Husain oversees the overall scientific and administrative leadership of the project. He oversaw the experimental plans and reviewed each of the data outcomes. He presides over the main meetings of the projects and directly interfaces with the CROs and other collaborators.

2. Mang Yu, MD, PhD

Project Role: Senior Research Scientist

Nearest person month worked: 12

Contribution to Project: Dr. Yu assisted Dr. Husain in overseeing and implementing the project. He also conducted mouse studies proposed in the proposal.

3. Rejowana Majid, MD

Project Role: Visiting Scholar

Nearest person month worked: 12

Contribution to Project: Dr. Majid performed surgical procedures to generate post-ERCP pancreatitis models.

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- Financial support;

- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

In this project year, we continued working with Premier Research, a regulatory CRO company, which offers strategic and tactical regulatory expertise in all phases of drug development. We would like to take advantage of their comprehensive experience to strategically guide us to achieve our drug development goals.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Appendix A

1) Significant results or key outcomes:

Preclinical safety evaluation of calcineurin inhibitors delivered through an intraductal route to prevent post-ERCP pancreatitis demonstrates endocrine and systemic safety

Jianbo Ni ^{a, b, 1}, Asna Khalid ^{a, 1}, Yu-Chu Lin ^{a, 1}, Monique T. Barakat ^{a, c}, Jing Wang ^d, Cheng-Yu Tsai ^a, Pasha Reza Shams Azar ^a, Ying Ding ^e, Judy-April Murayi ^f, Thottala Jayaraman ^g, Ronald Poropatich ^h, Rita Bottino ⁱ, Li Wen ^j, Georgios I. Papachristou ^k, Gayathri Swaminathan ^a, Mang Yu ^{a, **, *}, Sohail Z. Husain ^{a, *}

^a Division of Gastroenterology, Hepatology and Nutrition, Department of Pediatrics, Stanford University School of Medicine, Stanford, CA, USA

^b Department of Gastroenterology and Shanghai Key Laboratory of Pancreatic Disease, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

^c Division of Gastroenterology and Hepatology, Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA

^d Department of Radiology and Stanford Diabetes Research Center, Stanford University School of Medicine, Stanford, CA, USA

^e Department of Biostatistics, University of Pittsburgh, Pittsburgh, PA, USA

^f Division of Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics, Nationwide Children's Hospital, Columbus, OH, USA

^g Department of Oral and Craniofacial Sciences, School of Dental Medicine, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA

^h University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA

ⁱ Imagine Islet Center, Imagine Pharma, 1401 Forbes Avenue, Pittsburgh, PA, USA

^j Peking Union Medical College Hospital, Chinese Academy of Medical Science & Peking Union Medical College, Beijing, China

^k The Ohio State University Wexner Medical Center, Columbus, OH, USA

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ABSTRACT

Objective: There is an urgent need for safe and targeted interventions to mitigate post-ERCP pancreatitis (PEP). Calcineurin inhibitors (CnIs) offer therapeutic promise as calcineurin signaling within acinar cells is a key initiating event in PEP. In previous proof-of-concept studies using experimental models, we showed that concurrent intra-pancreatic ductal administration of the CnIs, tacrolimus (Tac) or cyclosporine A (CsA) with the ERCP radiocontrast agent (RC) prevented PEP. To translate this finding clinically, we investigated potential toxic effects of intraductal delivery of a single-dose RC-CnI formulation on endocrine pancreas function and systemic toxicities in a preclinical PEP model.

Methods: C57BL/6J mice underwent ductal cannulation and received a single, intra-pancreatic ductal infusion of RC or RC with Tac or CsA (treatment groups) or underwent ductal cannulation without infusion ('sham' group). To assess endocrine function, intraperitoneal glucose tolerance test (IPGTT) was performed at two days before infusion and on day 2 and 14 post-surgery. To evaluate off-target tissue toxicities, renal and hepatic function-related parameters including blood urea nitrogen, plasma creatinine, potassium, aspartate aminotransferase, alanine aminotransferase, and total bilirubin were measured at the same time-points as IPGTT. Histological and biochemical indicators of pancreas injury and inflammation were also evaluated.

Results: No abnormalities in glucose metabolism, hepatic or renal function were observed on day 2 or 14 in mice administered with intraductal RC or RC with Tac or CsA.

Conclusion: Intraductal delivery of RC-CnI formulation was safe and well-tolerated with no significant acute or subacute endocrine or systemic toxicities, underscoring its clinical utility to prevent PEP.

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* Corresponding author. Pediatric Gastroenterology, 750 Welch Road, Ste 116, Stanford, CA, 94305, USA.

** Corresponding author. Pediatric Gastroenterology, 1701 Page Mill Road, Ste 260, Palo Alto, CA, 94304, USA.

E-mail addresses: yumyu@stanford.edu (M. Yu), sohail.husain@stanford.edu (S.Z. Husain).

¹ co-first authors.

1. Introduction

1.1. PEP is a significant adverse event in ERCP procedures

Endoscopic retrograde cholangiopancreatography (ERCP) is a lifesaving, gastrointestinal procedure used commonly for both diagnosis and treatment of numerous pathologies in the pancreas, gallbladder, and liver. This procedure involves endoscopic cannulation of the biliary or pancreatic ducts followed by administration of a radiocontrast (RC) dye for intraluminal visualization prior to intervention. Post-ERCP pancreatitis (PEP) is a common adverse event in ERCP procedures with clinical features characteristic of acute pancreatitis including severe abdominal pain with concomitant increase in serum amylase and lipase more than three times the upper normal limit. Symptoms typically manifest within 24 h post ERCP and patients often require prolonged hospitalization. PEP has an incidence rate of 3–15% and is an important public health problem with an estimated 500,000 ERCP procedures being performed annually in the United States. PEP has been attributed to procedural pancreatic injury due to RC injection and/or irritation of the ampulla of Vater, leading to pancreatic ductal hypertension as well as pre-existing risk factors such as young age, female gender, sphincter of Oddi dysfunction, or prior PEP [1–8]. Current clinical interventions for reducing PEP including pancreatic stent placement, sublingual nitrates, IV fluid administration, somatostatin, NSAIDs (e.g., indomethacin) offer varying degrees of protection and is guided by patient risk for developing PEP. However, their usage is associated with added risks, significant side-effects, limited therapeutic window, long-term morbidities or high cost [9,10]. There is an urgent need for improved, cost-effective interventions to prevent PEP and associated long-term morbidity and mortality, with minimal side-effects. Identification of novel, targeted prophylactic agents that inhibit key molecular/cellular mechanisms involved in inducing and transducing PEP is therefore of immense clinical value.

1.2. Rationale for safety evaluation of intraductal RC-CnI formulations for PEP prevention

Previous studies from our laboratory showed that calcineurin (Cn), a serine/threonine protein phosphatase, is activated in pancreatic acinar cells in response to increased calcium influx early in the inflammation process and is a critical mediator of PEP [11]. Cn signaling augmented pancreatic inflammation via activation of transcription factors, nuclear factor of activated T cells (NFAT), and nuclear factor kappa B subunit 1 (NF- κ B), known contributors to pancreatitis pathogenesis and cellular necrosis. The prophylactic use of calcineurin inhibitors (CnIs) can therefore be envisioned to reduce incidence of PEP. In support of this premise are our proof-of-concept studies in experimental mouse models, which demonstrated that deletion of Cn in the pancreas or intraductal administration of RC with FDA-approved CnIs, tacrolimus (Tac/FK506) or cyclosporine A (CsA), was effective in preventing PEP [12]. To enable clinical translation of these encouraging results, toxicities commonly encountered with oral/systemic CnI use in transplant patients involving the kidney, liver and pancreas need to be evaluated [13–21]. Moreover, intraductal delivery causes direct pancreatic exposure to CnI that may result in toxic effects in the 'local' environment, not previously reported in CnI safety studies.

In the current study, we therefore aimed to investigate whether intraductal delivery of a single dose CnI formulation resulted in detrimental side-effects on endocrine pancreas function and off-target nephro- and hepato-toxicities in a preclinical model of PEP. We demonstrate that intraductal administration of CnIs is safe, with no unfavorable, coincident perturbation/impairment of

endocrine pancreas, liver or kidney function.

2. Materials and methods

2.1. Experimental animals

Wild type C57BL/6J mice, 10–12 weeks of age (Jackson Laboratory, Bar Harbor, ME) were used for the experiments. All mice were housed at 22 °C with a 12-h light-dark cycle and maintained on a standard laboratory chow diet with free access to food and water. All animal care and experiments were conducted in compliance with the guidelines of the National Institutes of Health and with specific approval from the Institutional Animal Care and Use Committee of Stanford University.

2.2. Intra-pancreatic ductal infusion of RC and Cn inhibitor formulations

Intra-pancreatic ductal infusion was performed in mice following a method described previously [22] with minor modifications. Briefly, anesthesia was induced and maintained with 2%–4% isoflurane using a vaporizer (E-Z Systems, Palmer, PA) and gas scavenger system (Patterson Scientific, Waukesha, WI). Using sterile technique, a small midline laparotomy window was created. The distal common bile duct was accessed via a trans-duodenal puncture and gentle intraluminal cannulation of the ampulla of Vater with a blunt-ended catheter. Retrograde biliopancreatic ductal or intraductal infusion of 0.1 mL radiocontrast agent (Omnipaque-240, GE Healthcare, Chicago, IL) containing 1 mM tacrolimus (Tac, Prograf, Astellas, Northbrook, IL) or 1 mM cyclosporine A (CsA, Sandimmune, Novartis, East Hanover, NJ) was performed at a speed of 4 μ L/s using a peristaltic rate-regulated pump (Harvard Apparatus, Holliston, MA). The proximal common bile duct was clamped so that the infusate flowed only into the pancreas. Mice from the 'sham' group underwent pancreatic duct cannulation without infusion. Intraperitoneal injection of buprenorphine (0.05 mg/kg) was given immediately after the surgery. The mice were monitored post-surgery for changes in physical activity, grooming routines, level of alertness, response to stimulation and other signs of distress.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from mice on day 2 and 14 after intra-pancreatic ductal infusion of RC containing 1 mM Tac or 1 mM CsA. Samples were centrifuged at 3000 rpm for 10 min at room temperature to collect serum. The levels of Tac and CsA in the serum were measured using a commercial ELISA kit (MyBioSource, San Diego, CA) following manufacturer's instructions. Absorbance (standards and test samples) was measured at a wavelength of 450 nm using the SpectraMax iD3 plate reader (Molecular Devices, San Jose, CA) and the serum levels of Tac and CsA were interpolated from a standard curve.

2.4. Intraperitoneal glucose tolerance testing (IPGTT)

IPGTT was performed 2 days before (for baseline estimate) and on day 2 and 14 after the intra-pancreatic ductal infusion in the treated group or control 'sham' group following the method described by Heikkinen and colleagues [23]. Briefly, the mice first underwent a 4-h food fast with access only to plain water. The Accutrend Plus system (Roche Diagnostics, Rotkreuz, Switzerland) was used to prick the tail vein. Blood glucose levels were measured before, and 15, 30, 60, 90, and 120 min after intraperitoneal injection of a dextrose solution (administered in normal saline at 2 g/kg).

During this procedure, mice were handled gently to avoid stress-induced hyperglycemia [24].

2.5. Blood tests to assess hepatic and renal function

Blood samples were collected from mice on day 2 and 14 after intra-pancreatic ductal infusion or sham procedure. Plasma levels of creatinine, potassium, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin and blood urea nitrogen (BUN) were measured by the Veterinary Services Center at Stanford University School of Medicine (Stanford, CA).

2.6. Plasma amylase and IL-6 measurement

Blood samples were collected from mice on day 2 and 14 after intra-pancreatic ductal infusion or sham procedure. Plasma amylase was measured using the Phadebas amylase test kit (Phadebas Inc, Cambridge MA). IL-6 levels were measured using an IL-6 mouse uncoated enzymelinked immunosorbent assay (Thermo Fisher Scientific/Invitrogen, Waltham, MA).

2.7. Histology

Mice were sacrificed 14 days after the intra-pancreatic ductal infusion (or sham) procedure. Pancreatic, renal, and hepatic tissues were fixed in 10% formalin for 24 h, embedded in paraffin, and sectioned into 4 μ m slices for hematoxylin-eosin (H&E) staining using standard procedures at Peninsula Histopathology Laboratory, Inc. (Campbell, CA). H&E-stained sections were scanned on an Aperio digital slide scanner (Leica Biosystems) using a 20 $\times$ objective and image analysis was done using the ImageScope software. Alternatively, images were captured using an Olympus microscope and cellSens software (Olympus LS). Histological scoring of pancreas for edema, inflammation, and necrosis was performed following the method described by Moreno and colleagues [25] with modifications (see Table 1). Histology scoring of liver sections for vacuolation, inflammation, and necrosis was done following the method described by Dai and colleagues [26]. The analysis was unbiased and blinded to the experimental groups.

2.8. Statistical analysis

All data are presented as mean $\pm$ SEM. Unless otherwise specified, differences among experimental groups were assessed by using two-way ANOVA, followed by Tukey's multiple comparison test using Prism 9.0 software (GraphPad, La Jolla, CA). A *P* value of less than 0.05 was considered statistically significant. Each experiment reported in this manuscript was repeated at least twice to confirm its reproducibility, which generated similar results.

3. Results

Intra-pancreatic ductal delivery of single dose, combined RC-Cnl formulations does not perturb endocrine pancreas function in a preclinical model of PEP. Cnl use in transplant patients has been associated with increased islet cell apoptosis, decreased beta-cell mass, impaired insulin secretion and disturbances in glucose metabolism. Furthermore, long-term Cnl use is considered a predisposing factor for development of post-transplant diabetes mellitus (PTDM) [13,27]. We therefore tested if intraductal administration of Cnls disrupted pancreas islet function and glucose homeostasis in a preclinical model of PEP. Wild-type C57BL/6J mice (10–12 weeks of age, male and female) were used to model PEP and assigned to three 'treatment' groups or 'sham' group. Mice assigned to treatment groups underwent pancreatic

duct cannulation and were given an intra-pancreatic ductal infusion of either RC agent (to simulate human PEP etiology) or RC agent with added Cnl, Tac or CsA (designated RC, RC + Tac and RC + CsA, respectively). Control mice ('sham' group) underwent pancreatic ductal cannulation without RC or Cnl infusion (Fig. 1A). Previous studies from our laboratory demonstrated that intraductal exposure to 10 μ M CsA (0.048 mg/kg body weight) and 1 μ M Tac (0.0032 mg/kg body weight) along with the RC agent was effective for PEP prevention in mice [12]. In the current study designed to test the safety of a single dose, intraductal infusion of Cnl in mice, we therefore tested a high concentration; 100-fold (4.8 mg/kg body weight) and 1000-fold (3.2 mg/kg body weight) higher for CsA and Tac, respectively. These doses provided measurable serum levels of Tac and CsA on day 2 and 14 post intraductal infusion (day 2: Tac 2.369 ± 0.1377 ng/ml; CsA 449.8 ± 33.95 ng/ml and day 14: Tac 1.934 ± 0.1207 ng/ml; CsA 492 ± 23.8 ng/ml CsA) (Supplementary Fig. 1) and was well-tolerated while achieving our scientific objective. In addition, we also considered the lethal doses reported with systemic and oral administration of Tac and CsA in mouse and rat [28], while estimating the intraductal dose used in this study.

To assess perturbation of endocrine pancreas function and glucose metabolism, we performed intraperitoneal glucose tolerance test (IPGTT) at 3 different time-points: two days pre-infusion to get a baseline estimate and on day 2 and 14 post-infusion in the treatment and sham groups. We chose day 2 as the early time-point to assess safety in order to allow the animals to recover from the stress of ductal cannulation surgery which can cause fluctuations in glucose levels independent of Cnl effects. Testing on day 14 was done to record any delayed manifestation of toxic effects due to direct pancreas exposure to the single dose formulations in accordance with FDA safety guidelines for an investigational new drug (IND). To measure temporal changes in blood glucose levels, maximal blood glucose concentration and glucose AUC (index for glucose intolerance), blood was drawn from the tail vein of mice at baseline and at 30, 60 and 120 min after injection (i.p.) of dextrose. Our results show that both the treatment and sham groups have a similar ability to metabolize glucose. Mice administered with RC + CsA showed a statistically significant increase ($P < 0.05$) in maximal blood glucose values when compared to the sham group on day 2 post procedure and to the RC treatment group on day 14 post procedure. However, the RC + CsA treated mice did not show any significant increase in maximal blood glucose compared to their baseline levels (Fig. 1B–D). Our results thus indicate that intraductal delivery of RC with Cnls does not cause any functional perturbation of endocrine pancreas and whole-body glucose homeostasis.

Intra-pancreatic ductal delivery of single dose RC-Cnl formulations does not cause any hepato- or nephrotoxicity. Toxicities involving the liver and kidney are the most documented non-pancreatic adverse effects associated with clinical use of Cnls [21]. However, the adverse events are typically linked to high doses, high variability in Cnl exposure or chronic use of Cnls. We hypothesized that single dose Cnl formulations delivered directly to the pancreas would have negligible off-target, systemic side-effects. To test this, we evaluated the plasma levels of hepatic and renal function-related parameters following intraductal administration of Cnls in the treatment (RC, RC + Tac, RC + CsA) and the 'sham' mice groups. The analysis was done at the same time-points as those used for IPGTT. We measured the levels of traditional markers of renal function including blood urea nitrogen (BUN), plasma creatinine, and potassium [29,30] and of liver function such as ALT, AST, and total bilirubin [31,32]. We observed no significant differences in renal and hepatic parameters across different treatment and sham mice groups on day 2 or day 14 post procedure as well as compared to baseline levels measured in naïve mice (Fig. 2A

Table 1
Histology scoring of pancreatitis.

Phenotype	Score	Morphological changes
Edema	0	absent
	1	focally increased between lobules
	2	diffusely increased between lobules
	3	acini disrupted and separated
Inflammation (leukocyte infiltration)	0	absent
	1	rare or around ductal margins
	2	diffusely increased between lobules
	3	acini disrupted and separated
Necrosis	0	absent
	1	architectural changes, pyknotic nuclei
	2	focal necrosis (<10% of the parenchyma)
	3	Diffuse parenchymal necrosis (>10% of the parenchyma)

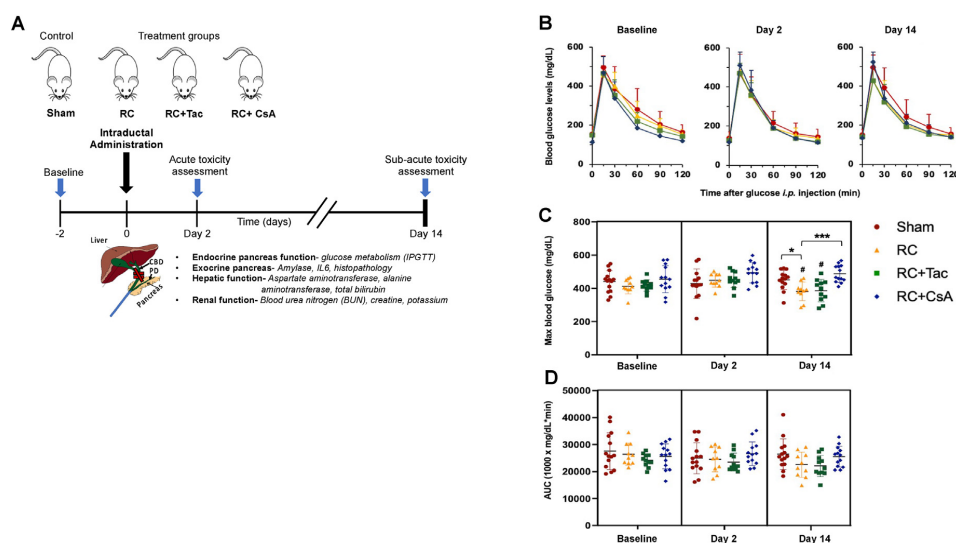


Fig. 1. Intra-pancreatic ductal infusion of novel radiocontrast (RC) and Cn inhibitor formulations does not disrupt glucose metabolism in a preclinical model of PEP. (A) Experimental groups, time-points and analysis scheme for acute and subacute toxicity assessment of RC-CnI formulations. Mice assigned to treatment groups underwent pancreatic duct cannulation and intra-pancreatic ductal infusion of RC or RC + Tac or RC + CsA. Mice in the 'sham' group underwent ductal cannulation without infusion. (B) Blood glucose levels over time, (C) maximal blood glucose concentrations and (D) glucose area under the curve (AUC) were measured 2 days pre-infusion (baseline) and on day 2 and 14, post-infusion or sham procedure. N = 10 to 13 mice per group. * $P < 0.05$ for maximal blood glucose day 14 versus day 2 post procedure * $P < 0.05$, *** $P < 0.001$ for group comparisons on day 14 as indicated. Two-way Anova with Tukey's post-test.

and B). Furthermore, comparison of day 2 and 14 values for each group revealed significantly lower ALT and total bilirubin levels in the RC + CsA group but no other groups (Fig. 2B). No statistical differences were noted in the levels of other blood markers of renal and hepatic function in the treatment and sham groups (Fig. 2A and B).

To complement the blood correlates of renal and hepatic function, we examined H&E-stained kidney and liver tissue sections on day 14 after intraductal CnI administration for evidence of tissue level changes/toxicity. For kidney, we evaluated for signs of vacuolation in the proximal tubular cells (noted with CnI and RC use), tubular necrosis, infiltration of immune cells, changes in glomeruli, and vascular damage. For liver, we surveyed H&E tissue sections for hepatocellular necrosis, injury-related morphological changes around the portal areas, loss of lobular architecture, and immune cell infiltration. We did not observe overt changes to the renal and hepatic parenchyma or tissue inflammation resulting from the Tac or CsA treatment, consistent with the lack of change in the

biochemical parameters of renal and hepatic function (Supplementary Figs. 2A–B and Fig. 2). However, we did observe occasional disturbance in the liver parenchyma, with the frequency and extent of changes being similar in all the experimental groups (Supplementary Fig. 2C) and thus, this is likely an artifact of the bile duct cannulation model required for intraductal infusion. Importantly, these changes did not affect hepatic functional parameters. Together, our results demonstrate that intraductal administration of RC-CnI was well-tolerated with no significant acute or sub-acute renal or hepato-toxicities.

Intra-pancreatic ductal administration of single dose RC-CnI does not exacerbate pancreatic injury and inflammation associated with PEP. We previously reported that intraductal infusion of RC-CnI combination in experimental models reduced PEP-associated pancreas tissue damage and inflammation [12]. The intraductal administration of CnIs, unlike the systemic/oral route, causes direct exposure of pancreas to CnI. This may result in acute or delayed, subacute toxic effects to pancreatic tissue and

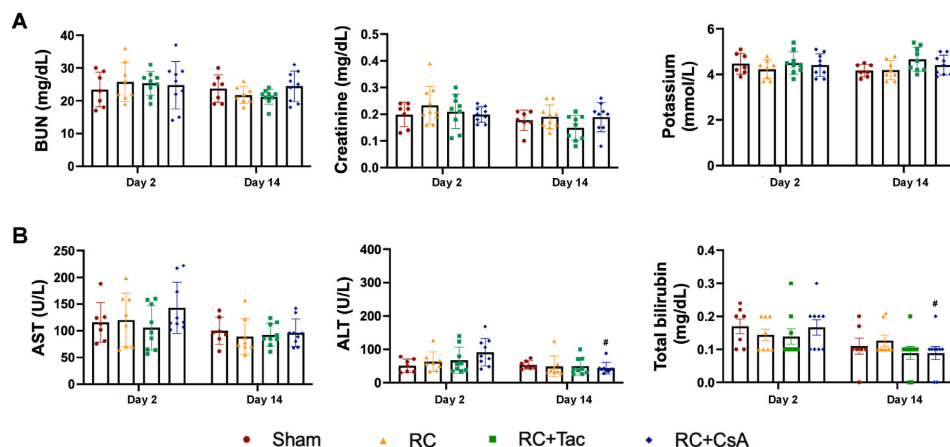


Fig. 2. Intra-pancreatic ductal delivery of RC-CnI combination does not cause renal or hepatic toxicity/functional abnormalities. Plasma levels of (A) renal function parameters including blood urea nitrogen (BUN), creatinine, potassium and (B) hepatic function parameters including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin were measured in different experimental groups on day 2 and 14 post sham procedure or intra-ductal infusion of RC or RC-CnI combination as indicated. N = 7 to 9 mice per group. * $P < 0.05$; value on day 14 compared to the corresponding value on day 2 post procedure.

impairment of exocrine/endocrine function. Our results reported in Fig. 1 demonstrate no significant changes in whole body glucose metabolism, suggestive of normal endocrine pancreas function following intraductal delivery of RC-CnI formulations in the pre-clinical PEP models. To ascertain effects on exocrine pancreas, we evaluated histopathological features as well as biomarkers of pancreatic injury and inflammation. H&E-stained pancreas sections were scored for edema, inflammation and necrosis on day 14 post intraductal administration of CnIs in the treatment (RC, RC + Tac, RC + CsA) groups and sham mice group. We observed that the RC + Tac and RC + CsA treatments groups showed significant decrease in tissue edema and necrosis compared to mice treated with RC alone, with Tac showing superior effects to CsA. Tac but not CsA administration was also associated with markedly reduced pancreas inflammation (Fig. 3A–C). In addition, we specifically examined the pancreatic duct cells for evidence of toxic effects as they are likely to see the highest drug concentration following intraductal administration. An unbiased, blinded assessment of H&E-stained pancreas tissue sections revealed that the duct epithelial cells remained unaffected by the Tac and CsA treatment with no overt changes in morphology or organization (Supplementary Fig. 3).

Further analysis of plasma levels of amylase (biomarker of pancreatic injury) and the pro-inflammatory cytokine, IL-6 showed a marked increase in RC, RC + CsA treated and sham mice on day 2 compared to baseline levels of amylase (3.2 ± 0.5 U/ml) or IL-6 (18.4 ± 2.5 pg/ml) measured in 'naïve' mice two days prior to the procedure (RC > sham > RC + CsA; Fig. 3H and I). The RC + Tac group, however, did not show significant increase in amylase or IL-6 levels over baseline. This is consistent with the markedly reduced tissue injury and inflammation in the RC + Tac group compared to the RC group in our study. We also observed that concurrent Tac or CsA delivery with RC was effective in minimizing amylase and IL-6 increases on day 2 seen with RC alone. Additional measurements of amylase on day 14 revealed restoration to baseline levels in all experimental groups compared to their day 2 counterparts. Unlike amylase, IL-6 levels remained elevated over baseline in the sham and RC treated mice, while showing considerable decline in the Tac and CsA treated mice. (Fig. 3I). Thus, our results indicate that

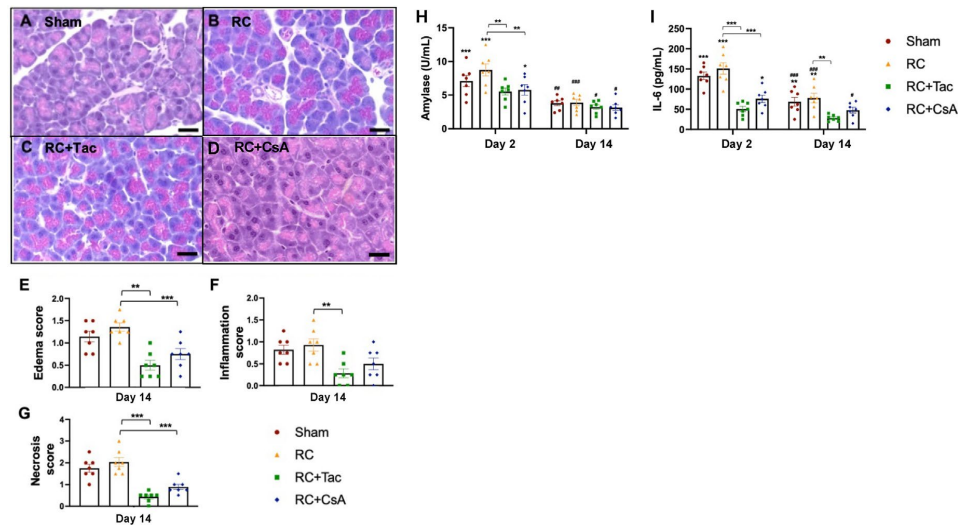
despite 'direct' pancreas exposure to much higher concentration of both Tac (1 mM) and CsA (1 mM) compared to that used in our previous studies (Tac 1 μ M, CsA 10 μ M) [12], no overt acute or delayed exocrine toxicity was discernible, underscoring the safety of both CnI agents and the intraductal delivery route.

4. Discussion

In this IND enabling study, we show that intraductal delivery of CnIs with the RC contrast dye during an ERCP procedure is safe and well-tolerated in a preclinical model of PEP. No evidence of acute or sub-acute toxic side-effects on endocrine/exocrine pancreas, kidney or liver function were noted. Interestingly, the unique single dose formulation of RC-CnI especially, tacrolimus, had a prophylactic effect against PEP with significantly reduced pancreatic injury and inflammation post-procedure. Our findings present evidence-based rationale for single dose, intraductal application of the FDA-approved CnIs (Tac or CsA) in combination with RC as a safe, targeted intervention for PEP with minimal side-effects during ERCP procedures.

Post-transplantation diabetes mellitus (PTDM) remains an important complication associated with clinical use of CnIs for immune suppression in transplantation recipients. However, it is often reversible with CnI dosage reduction or withdrawal [14,33]. In order to separate the acute effects on glucose metabolism due to RC-CnI administration from those caused by procedural isoflurane anesthesia and surgery-related acute stress [34,35], IPGTT was performed on day 2 post-procedure. No significant changes were noted. However, we cannot rule out the possibility of acute abnormalities in glucose tolerance occurring at earlier time points after CnI administration in our model. It is known that effects of CnI on glucose metabolism is subject to temporal fluctuations; hyper-acute changes in our model may have already been resolved by the time of IPGTT on day 2.

Sporadic cases of pancreatitis associated with Tac use in renal, and umbilical cord blood transplant patients have been previously reported [36–39]. Symptoms were reversible in most cases with drug withdrawal. Pancreatitis has also been reported as a rare adverse event in transplant patients treated with CsA. Thus, it is



y 2 post procedure. N = 7 mice per group.

possible that direct exposure of pancreas to Tac/CsA delivered via the intraductal route can synergize with PEP and exacerbate disease symptoms in some patients. However, this scenario seems unlikely considering that the single dose RC-CnI formulations used in our preclinical model designed to test safety was in fact associated with significantly reduced signs of exocrine pancreas injury and was protective against PEP. Further, the CnI-induced pancreatitis in the reported instances were likely due to high dose and systemic use in the context of immune suppression in transplant patients, unlike the single dose intraductal CnI delivery for PEP prophylaxis tested in our study. In this context, it is worth noting emerging data from a large, retrospective study in liver transplant patients on Tac and a prospective pilot study in patients with malignant/non-malignant indications (not transplant related), given two doses of oral Tac that showed substantial decrease in PEP incidence and severity [40–42]. These studies support our pre-clinical data on the safety and feasibility of therapeutic use of CnI in ERCP procedures to prevent PEP.

To monitor for potential renal and liver function impairments caused by the intraductal infusion of Tac or CsA, we used traditional/standard clinical markers of renal dysfunction, including blood urea nitrogen (BUN), plasma creatinine, and potassium, as well as of hepatotoxicity such as plasma ALT, AST, and total bilirubin. Mice that received intraductal infusion of RC + Tac or RC + CsA did not show significant changes in the biomarker levels compared with mice that received RC infusion alone, demonstrating systemic safety of intraductal RC-CnI formulations. However, these plasma markers may not be fully sensitive to immediate hepatotoxic or nephrotoxic effects of CnI, especially at early stages of end organ dysfunction. Therefore, future investigation using additional, sophisticated measures are warranted.

ERCP procedure-induced edema of the ampulla of Vater as well as the radiocontrast infusion can induce an acute elevation of pancreatic ductal pressure [43,44], which contributes to post-

procedure complications, including pancreatitis [45–47]. In mice, it has been well established that elevated intrapancreatic ductal pressure can induce pancreatitis [22,48,49]. In the present study, mice that underwent intraductal cannulation with RC infusion or cannulation alone (sham) showed detectable increases in biomarkers of pancreatic injury and inflammation including plasma amylase and IL-6 even after 2 days of the surgery, associating the cannulation or cannulation plus infusion procedures to pancreatic inflammation and injury. The histopathological changes and inflammation associated with the pancreatic injury were evident even after 14 days post procedure. However, this inflammatory state did not cause renal or liver biomarker abnormalities throughout the 2-week post-procedure period.

CnIs have been shown to modulate the levels of pro- or anti-inflammatory cytokines in a context dependent manner. For instance, treatment with Tac decreased IL-6 levels in human PBMC stimulated with anti-CD3/CD28 antibodies and in rheumatoid arthritis (the source of IL-6 being T-cells), while it increased IL-6 levels in endothelial cells [50–52]. In our preclinical model of PEP designed to study safety of intraductal CnI, despite an exposure to a 1000-fold and 100-fold higher dose compared to the protective dose demonstrated in our previous studies (1 μ M Tac, and 10 μ M CsA) [12], we did not observe any detrimental effects on exocrine pancreas or increase in IL-6 levels compared to the sham or RC group. In fact, we observed a protective effect of the CnIs, with a significant decrease in pancreas injury and lower serum IL-6 levels (day 2 and 14) in the CnI-treated groups (RC + Tac and RC + CsA) compared to the untreated groups (sham and RC), which showed signs of pancreatic injury. Thus, it appears that local, targeted intraductal delivery of CnIs mitigates the pro-inflammatory effects to protect pancreas from PEP. Considering experimental data that Cn signaling is a critical early event in AP (beyond the etiology of PEP), it is possible that the anti-inflammatory effects of CnIs (evidenced in the PEP model) can be capitalized for AP prevention.

Future studies are warranted to test the broad applicability of anti-inflammatory effects of CnIs in AP due to different etiologies.

There is currently no intraductal preventive for PEP. Single dose intraductal delivery of CnI such as Tac during ERCP procedures is likely to circumvent toxicities associated with systemic delivery. Our encouraging safety data on single dose RC-CnI formulations delivered directly to the pancreas for PEP prophylaxis offers immense therapeutic promise by repositioning a well-characterized, FDA approved, widely available, cost-effective drug for PEP prevention. To accelerate clinical delivery, evaluation of pharmacokinetics of CnIs following intraductal delivery and carefully controlled efficacy testing of intraductal CnI formulations as monotherapy or in combination with other agents such as indomethacin is warranted.

Declaration of competing interest

S.Z.H is on the Scientific advisory board of ATIAS pharma.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pan.2023.03.009>.

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Rectal administration of tacrolimus protects against post-ERCP pancreatitis in mice

Yu-Chu Lin ^{a,1}, Jianbo Ni ^{a,b,1}, Gayathri Swaminathan ^a, Asna Khalid ^a,
Monique T. Barakat ^{a,c}, Adam R. Frymoyer ^d, Cheng-Yu Tsai ^a, Ying Ding ^e,
Judy-April Murayi ^f, Thottala Jayaraman ^g, Ronald Poropatich ^h, Rita Bottino ⁱ, Li Wen ^j,
Georgios I. Papachristou ^k, Sunil G. Sheth ^l, Mang Yu ^{a,*}, Sohail Z. Husain ^{a,*}

^a Division of Gastroenterology, Hepatology and Nutrition, Department of Pediatrics, Stanford University, School of Medicine, Stanford, CA, USA

^b Department of Gastroenterology and Shanghai Key Laboratory of Pancreatic Disease, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

^c Division of Gastroenterology and Hepatology, Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA

^d Division of Neonatology, Department of Pediatrics, Stanford University School of Medicine, Stanford, CA, USA

^e Department of Biostatistics, University of Pittsburgh, Pittsburgh, PA, USA

^f Division of Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics, Nationwide Children's Hospital, Columbus, OH, USA

^g Department of Oral and Craniofacial Sciences, School of Dental Medicine, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA

^h University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA

ⁱ Imagine Islet Center, Imagine Pharma, 1401 Forbes Avenue, Pittsburgh, PA, USA

^j Institute of Clinical Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Science & Peking Union Medical College, Beijing, China

^k The Ohio State University Wexner Medical Center, Columbus, OH, USA

^l Beth Israel Deaconess Medical Center, Boston, MA, USA

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ABSTRACT

Objective: There is an unmet clinical need for effective, targeted interventions to prevent post-ERCP pancreatitis (PEP). We previously demonstrated that the serine-threonine phosphatase, calcineurin (Cn) is a critical mediator of PEP and that the FDA-approved calcineurin inhibitors, tacrolimus (Tac) or cyclosporine A, prevented PEP. Our recent observations in preclinical PEP models demonstrating that Cn deletion in both pancreatic and hematopoietic compartments is required for maximal pancreas protection, highlighted the need to target both systemic and pancreas-specific Cn signaling. We hypothesized that rectal administration of Tac would effectively mitigate PEP by ensuring systemic and pancreatic bioavailability of Tac. We have tested the efficacy of rectal Tac in a preclinical PEP model and in cerulein-induced experimental pancreatitis.

Methods: C57BL/6 mice underwent ductal cannulation with saline infusion to simulate pressure-induced PEP or were given seven, hourly, cerulein injections to induce pancreatitis. To test the efficacy of rectal Tac in pancreatitis prevention, a rectal Tac suppository (1 mg/kg) was administered 10 min prior to cannulation or first cerulein injection. Histological and biochemical indicators of pancreatitis were evaluated post-treatment. Pharmacokinetic parameters of Tac in the blood after rectal delivery compared to intravenous and intragastric administration was evaluated.

Results: Rectal Tac was effective in reducing pancreatic injury and inflammation in both PEP and cerulein models. Pharmacokinetic studies revealed that the rectal administration of Tac helped achieve optimal blood levels of Tac over an extended time compared to intravenous or intragastric delivery.

Conclusion: Our results underscore the effectiveness and clinical utility of rectal Tac for PEP prophylaxis.

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* Corresponding author. Pediatric Gastroenterology, 750 Welch Road, Ste 216, Stanford, CA 94305, USA.

** Corresponding author. Pediatric Gastroenterology, 1701 Page Mill Road, Ste 260, Palo Alto, CA 94304, USA.

E-mail addresses: myu@jaspertherapeutics.com (M. Yu), sohail.husain@stanford.edu (S.Z. Husain).

¹ Co-first authors.

1. Introduction

1.1. PEP is a common and severe complication in ERCP procedures

Endoscopic retrograde cholangiopancreatography (ERCP) is a lifesaving, gastrointestinal procedure frequently performed for diagnosis and management of pancreaticobiliary disorders. Post-ERCP pancreatitis (PEP) is a common adverse event in ERCP procedures with an incidence rate of 3–15%. PEP is an important public health concern with an estimated 500,000 ERCP procedures being performed annually in the United States. The clinical manifestations of PEP including severe abdominal pain, and an increase in serum amylase and lipase more than three times the upper limit of normal, typically manifest 24 h post ERCP and patients often require prolonged hospitalization. Current clinical interventions for reducing PEP such as pancreatic stent placement, fluid resuscitation, non-steroidal anti-inflammatory drugs (NSAIDs, for e.g., indomethacin) offer varying degrees of protection and their usage is associated with added side-effects or high cost [1–9]. There is an unmet clinical need for improved, cost-effective, and targeted interventions to prevent PEP with minimal side effects.

1.2. Rationale for the therapeutic utility and rectal administration of the calcineurin inhibitor, tacrolimus for PEP prophylaxis

Previous studies from our lab using experimental mouse models demonstrated that the serine-threonine phosphatase, calcineurin (Cn) is a critical mediator of PEP [10]. Importantly, we showed that deletion of Cn in the pancreas or administration of the radio-contrast (RC) dye along with the FDA-approved calcineurin inhibitors (CnIs), tacrolimus (Tac/FK506) or cyclosporine A (CsA), was effective in preventing PEP [10,11]. Our results thus provided compelling evidence for the use of CnIs for PEP prophylaxis. Although our preclinical data suggests that both Tac and CsA are attractive candidates for PEP prevention, we propose to focus on Tac due to its higher specificity and potency as a CnI [12,13]. Furthermore, the feasibility of repurposing Tac for PEP prevention is supported by a large, retrospective clinical study which showed a significant decrease in PEP after ERCP procedures in liver transplant patients on a regular regimen of Tac for immunosuppression. This study demonstrated that a pre-procedure trough level of Tac above 2.5 ng/mL was associated with 79% lower odds for PEP [14]. Of note, the authors observed a significant, added therapeutic benefit with administration of a single dose of rectal indomethacin to patients with Tac trough levels >2.5 ng/mL, which decreased PEP incidence in this cohort from 2.5% to 0.4%. In addition, a recent pilot interventional study in patients with malignant and non-malignant indications (non-transplant related) undergoing ERCP demonstrated nearly a 50% decrease in PEP incidence among patients given two doses of oral Tac (4 mg total) as compared to patients who were not given Tac [15]. Taken together, both pre-clinical and clinical studies provide strong evidence-based rationale for the prophylactic use of Tac for PEP prevention.

An important consideration for clinical use of Tac for PEP prevention is the time, dose, and route of administration to ensure optimal delivery and bioavailability of therapeutic doses, while minimizing undesirable side effects inherent in the systemic use of Tac. We previously showed in preclinical PEP models that intraductal administration of Tac with the RC dye during the ERCP procedure was safe, well-tolerated and effective in PEP prevention [11,16]. However, our recent observations in preclinical models of PEP demonstrated that Cn deletion in both pancreatic and hematopoietic compartments is required for maximal pancreas protection. This highlighted the need to target both systemic and pancreas-specific Cn signaling for PEP prevention [17]. Another

consideration is that procedural improvements in ERCP have decreased the need for ductal contrast injection. Specifically, the development of guidewire based cannulation approaches has resulted in less than 10% of all ERCPs, and fewer than 25% of technically complex ERCPs (that confers high risk for PEP) undergoing RC injections [8]. We hypothesized that rectal administration of Tac prior to an ERCP procedure would ensure optimal pancreas and systemic bioavailability of Tac to mitigate pancreas injury and deliver favorable outcomes with minimal side effects. Rectal drug formulations are widely used clinically for both local and systemic indications [18], and may offer important advantages over conventional systemic routes (e.g., oral, or intravenous) for gastrointestinal (GI) indications. These include ease of administration, sustained drug release, local and systemic bioavailability, bypass of the upper GI digestion process as well as hepatic first-pass drug elimination which may improve increase bioavailability to target tissues with a lower dose, while reducing side-effects such as nausea and vomiting. Regarding rectal administration of Tac, there is encouraging clinical data that demonstrate both safety of rectal Tac suppositories and its effectiveness in achieving clinical remission and promoting mucosal healing in ulcerative proctitis patients resistant to conventional therapies [19,20]. However, it is not known whether Tac supplied as a rectal suppository would be effective in reducing PEP associated pancreatic injury and inflammation and whether it would have a favorable PK profile compared to other systemic routes (intravenous, oral) at doses which demonstrate disease alleviation.

In the current study, we therefore investigated whether rectal delivery of Tac protected the pancreas from injury and inflammation using two experimental models of acute pancreatitis, namely the pancreatic intraductal pressure-induced PEP model and the cerulein hyperstimulation model of AP.

2. Materials and methods

2.1. Experimental animals

Wild type C57BL/6J mice, 10–12 weeks of age, male and female (Jackson Laboratory, Bar Harbor, ME) were used for the experiments. All mice were housed at 22 °C with a 12 h light-dark cycle and maintained on a standard laboratory chow diet with free access to food and water. All animal care and experiments were conducted in compliance with the guidelines of the National Institutes of Health and with specific approval from the Institutional Animal Care and Use Committee of Stanford University.

2.2. Acute pancreatitis mouse models

2.2.1. Cerulein model of AP

C57BL/6J mice were given seven, hourly intraperitoneal injections of 50 µg/kg cerulein or Phosphate Buffered Saline (PBS; control) to induce pancreatitis [21]. For the rectal Tac treated experimental group, a rectal Tac suppository (1 mg/kg) was administered 10 min prior to the start of hourly cerulein injections.

2.2.2. PEP model

Intra-pancreatic ductal infusion was performed in mice following a method described previously [22], with minor modifications. Briefly, anesthesia was induced and maintained with 2%–4% isoflurane using a vaporizer (E-Z Systems, Palmer, PA) and gas scavenger system (Patterson Scientific, Waukesha, WI). Using sterile technique, a small midline laparotomy window was created. The distal common bile duct was accessed via a *trans*-duodenal puncture and gentle intraluminal cannulation of the ampulla of Vater with a blunt-ended catheter. Intraductal infusion of normal

saline was done for 10 min at a constant pressure of 75 mmHg. The proximal common bile duct was clamped so that the infusate flowed only into the pancreas. Mice from the 'sham' group underwent pancreatic duct cannulation without infusion. Intraperitoneal injection of buprenorphine (0.05 mg/kg) was given immediately after the surgery.

To deliver Tac rectally to the experimental pancreatitis groups, mice were administered a rectal suppository containing the excipient, Witepsol H-15 with or without Tac (1 mg/kg), in a volume of 20 μ L, 10 min prior to the start of surgery or the initial cerulein injection. The rationale for administration of Tac, 10 min prior to exposure to pancreatitis triggers in our preclinical model, was to simulate the clinical scenario of administering Tac prior to the start of ERCP, when patients are under anesthesia. The time of administration was further guided by clinical studies reporting lower incidence of post-ERCP pancreatitis with pre-procedure, prophylactic administration (within 30 min prior to start of ERCP) of a single dose (100 mg) of rectal indomethacin [23] or rectal diclofenac [24], compared to post-procedure administration. The mice were monitored post-surgery for changes in physical activity, grooming routines, level of alertness, response to stimulation and other signs of distress.

2.3. Plasma amylase and IL-6 measurement

Blood samples were collected from mice at 6, 12 and 24 h post cerulein injection or intrapancreatic ductal infusion or sham procedure. Plasma amylase was measured at all three time points using the Phadebas amylase test kit (Phadebas Inc, Cambridge, MA). IL-6 levels were measured at the 24 h time point using an IL-6 mouse uncoated enzyme linked immunosorbent assay (ThermoFisher Scientific/Invitrogen, Waltham, MA).

2.4. Histology

Mice were sacrificed at 24 h post-intrapancreatic ductal infusion (or sham) procedure or cerulein injections. Pancreatic tissues were fixed in 10% formalin for 24 h, embedded in paraffin, and sectioned into 4 μ m slices for hematoxylin-eosin (H & E) staining using standard procedures at Peninsula Histopathology Laboratory, Inc. (Campbell, CA). Histological scoring was performed following the method described by Moreno and colleagues [25], with modifications [16].

2.5. Tacrolimus formulations for IV, IG and rectal administration

To prepare the Tac rectal suppository, an aliquot of Prograf (Tac, 5 mg/mL, Astellas, Northbrook, IL) was added to the suppository base, Witepsol H15 (SpecializedRx, Fridley, MN) at 36 °C, to achieve a dosage of 1 mg Tac/kg/0.02 mL. The luer fitting with 2 cm-long soft polyvinyl chloride tubing from a Surflo winged infusion set was used for the solidification of the Tac suppository inside at room temperature as well as the following step of rectal delivery. For the intravenous or intragastric administration of Tac, an aliquot of Prograf was mixed with normal saline to achieve a dosage of 1 mg Tac/kg/0.1 mL.

2.6. Measurement of blood levels of Tac for pharmacokinetic (PK) analysis

Blood samples were collected from wild-type C57BL/6 mice prior to (0 h time point) and after IV, IG, and rectal (REC) administration of 1 mg/kg Tac (5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h time points). The blood levels of Tac were measured using a commercial ELISA kit (MyBioSource; San Diego, CA)

following manufacturer's instructions, for the analysis of PK parameters. Absorbance (standards and test samples) was measured at a wavelength of 450 nm using the SpectraMax iD3 plate reader (Molecular Devices; San Jose, CA).

2.7. Statistical analysis

All data are presented as mean $\pm$ SEM or mean + SEM. Unless otherwise specified, statistical significance was assessed by using one-way ANOVA (for experimental group comparisons) or two-way ANOVA (for experimental group and time comparisons), followed by Tukey multiple comparison test using Prism 9.0 software (GraphPad, La Jolla, CA). For each experimental group, the difference between two time points was further assessed by Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant. Each experiment reported in this manuscript was repeated at least twice to confirm their reproducibility, which generated similar results.

3. Results

3.1. Rectal administration of tacrolimus alleviates pancreatic injury and inflammation in experimental models of acute pancreatitis (AP)

Our previous studies in preclinical models of pancreatitis demonstrated the need to target both systemic and pancreas-specific Cn signaling for conferring maximal pancreas protection and provided strong evidence highlighting the potential clinical utility of the calcineurin inhibitor, Tac, for PEP prevention [11,17]. In this study we, therefore, tested the hypothesis that rectal administration of Tac would ensure optimal pancreas and systemic bioavailability of Tac to mitigate pancreas injury and deliver favorable outcomes, using two experimental models of AP namely, PEP and the cerulein-induced hyperstimulation AP.

To model PEP, pancreatitis was induced in C57BL/6J mice by elevating the intra-pancreatic ductal pressure. Mice were assigned to three experimental groups. The PEP and the PEP + REC Tac groups were administered a rectal suppository with either excipient alone or excipient with Tac (1 mg/kg), respectively, 10 min prior to pancreatic duct cannulation and intraductal infusion of normal saline at a pressure of 75 mmHg. The 'sham' control group underwent ductal cannulation without receiving any infusions or Tac treatment (Fig. 1a). For all experimental groups, we measured plasma amylase levels (marker of pancreas injury) at 6, 12 and 24 h, IL-6 levels (marker of pancreas inflammation) at 24 h, and performed histopathological scoring (edema, necrosis, and inflammation) of pancreas tissue H & E images at 24 h, post pancreatitis induction. Our results demonstrated that the PEP + REC Tac group had a significant early reduction in amylase levels at 6 h as compared to the PEP group. Furthermore, the mean levels of amylase were similar at 6 and 12 h (7.81 U/mL and 7.34 U/mL, respectively) and declined at 24 h (4.13 U/mL) in the PEP + Tac group as compared to the PEP group which showed elevated amylase levels at 6 h (14.3 U/mL) followed by a significant decline at 12 and 24 h (7.8 U/mL and 5.74 U/mL, respectively; Fig. 1b). In addition, the PEP + REC Tac group also showed a striking decrease in IL-6 levels (Fig. 1c) as well as histopathological scores at the 24 h time point as compared to the PEP group (Fig. 1d). However, the histopathological scores for the PEP + REC Tac group were higher than the control group suggesting that the dose of rectal Tac administered did not completely abrogate pancreas damage and is consistent with the modest increase in IL-6 levels in the PEP + REC Tac group compared to the control group.

We next evaluated the efficacy of rectal Tac in preventing pancreas injury and inflammation in the cerulein-induced

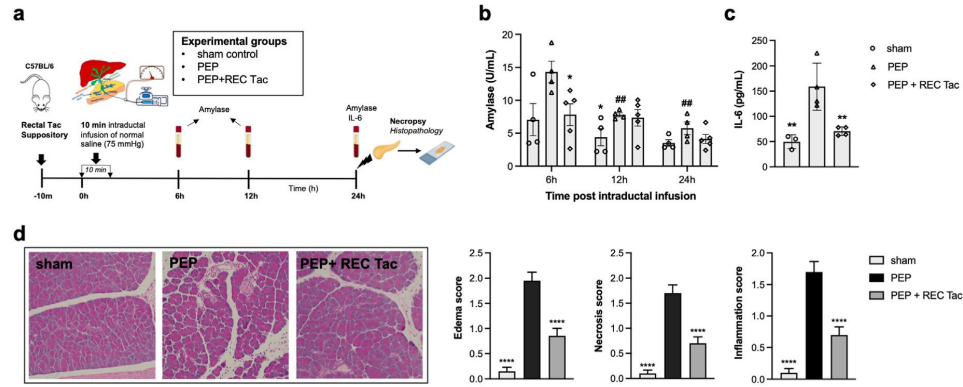


Fig. 1. Rectal Tac suppository is effective in reducing PEP associated pancreatic inflammation and tissue damage. (a) Preclinical PEP model to assess the effect of rectal Tac suppository (1 mg/kg) on PEP pathology. (b) Plasma levels of amylase, and (c) IL-6. (d) H & E images of pancreatic tissue and histopathology scores in the different experimental groups, as indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to PEP group; ## $P < 0.01$ compared to amylase levels at 6 h for the same experimental group; $n = 3-5$ mice per group. Baseline amylase levels in naïve mice = 1.147 U/mL.

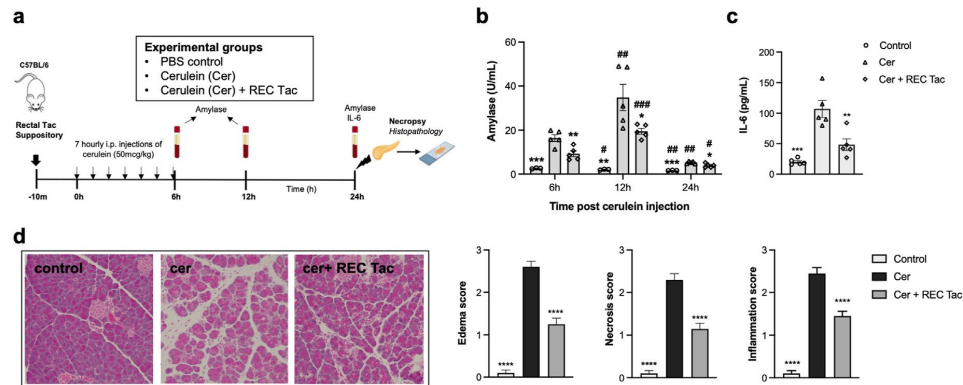
pancreatitis model. Control mice received seven, hourly PBS injections, while mice assigned to the cerulein (Cer) and cerulein + REC Tac (Cer + REC Tac) groups received seven, hourly cerulein injections. The latter two groups were administered a rectal suppository with either excipient alone or excipient with Tac (1 mg/kg), respectively, 10 min prior to the first cerulein injection (Fig. 2a). Analysis of plasma amylase, IL-6, and pancreas H & E images for histopathological scoring was done as described above for the PEP model. Our results showed that the levels of amylase and IL-6 as well as the histological parameters of pancreas injury and inflammation were significantly lower in the Cer + REC Tac group compared to the Cer group at the time points tested (Fig. 2b–d). However, as seen in the PEP model, rectal Tac did not completely prevent pancreatic injury and inflammation as the histology scores, amylase, and IL-6 values were higher than in control mice.

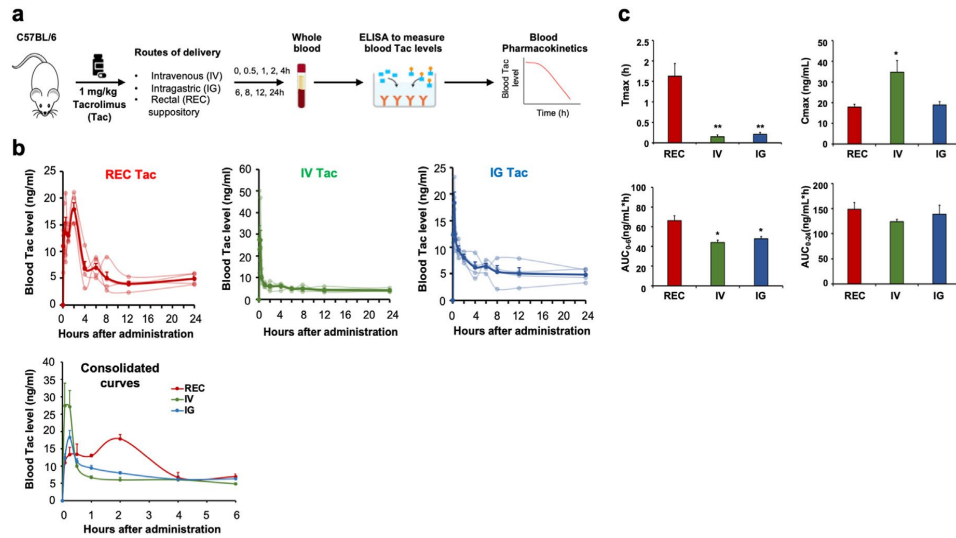
In sum, our findings in two experimental AP models demonstrate that administration of rectal Tac prior to pancreatitis induction can protect the pancreas from injury and inflammation, as

evidenced by the significant decrease in pancreatitis-associated biochemical and histopathological parameters.

3.2. Rectal delivery of tacrolimus (Tac) achieves optimal and durable blood levels of Tac

Given our encouraging results that a single-dose of rectal Tac was capable of ameliorating PEP and cerulein-induced pancreatitis, we determined the PK parameters of the rectal Tac suppository compared to intravenous (IV) and intragastric (IG) dosing in wild-type C57BL/6J mice. Following Tac administration, blood was collected at several timepoints to measure Tac levels in the blood over time and for calculating the PK parameters including the time to achieve maximum blood Tac levels (T_{max}), the maximum blood concentration of Tac (C_{max}) and total Tac exposure as measured by area under the concentration-time curve (AUC; 0–6 h and 0–24 h), post dosing (Fig. 3a). Our analysis of blood Tac concentration over time showed that the delivery through the rectal route resulted in slower systemic peaking of Tac levels with higher mean T_{max}





very of 1 mg/kg Tac. * $P < 0.05$, ** $P < 0.01$ versus REC group; $n = 3-5$ mice per group.

values (1.63 h) compared to the IV (0.15 h) and IG (0.21 h) route. Furthermore, the mean Cmax values for rectal Tac (17.93 ng/mL) was comparable to that achieved by Tac delivered through the IG route (18.84 ng/mL) but considerably lower than levels after IV administration (34.6 ng/mL). However, despite the delayed peaking and lower Cmax, rectal Tac had a favorable AUC profile as evidenced by significantly higher mean AUC₀₋₆ (measure of short-term drug exposure after dosing) and modestly higher mean AUC₀₋₂₄ (measure of long-term drug exposure after dosing) values as compared to both the IG and the IV routes (Fig. 3b and c and Table 1). Our PK data compare favorably to reported PK values for different Tac formulations and routes of administration in different rodent species and humans (Table 1) [26–30]. Our results thus demonstrate that the delivery of a single dose of Tac (1 mg/kg) as a rectal suppository provides optimal Tac exposure and can achieve therapeutic levels of Tac in the blood favorable for PEP prevention.

4. Discussion

PEP presents a significant iatrogenic challenge in ERCP. Current prophylactic measures show variable effectiveness and, in some cases, cause additional complications. The utility of the FDA approved CnI, Tac in preventing PEP incidence and severity, through Cn blockade, is underscored by both preclinical and clinical studies. Our current proof-of-concept preclinical study is the first to demonstrate the effectiveness of single-dose rectal Tac suppository in reducing PEP pathology. Taken together with the favorable PK profile of Tac delivered rectally as compared to intravenous and oral routes, our results make a strong case for the potential clinical use of rectal Tac for PEP prevention. Our findings in preclinical PEP models, clinical data showing favorable PK profiles with rectal Tac administration in healthy human subjects as well as clinical observations on the efficacy and safety of rectal Tac suppository in refractory ulcerative proctitis or colitis, provide a strong foundation to launch in-depth preclinical and clinical safety/efficacy studies for prophylactic use of single-dose rectal Tac during ERCP procedures

as monotherapy or in combination with NSAIDs such as indomethacin or diclofenac for PEP prevention. This treatment modality may also find clinical use in the potential management of other GI inflammatory disorders, where Cn blockade is desired. We are further encouraged by the clinical success of rectal indomethacin for PEP prevention in both high and average risk subjects undergoing ERCP [31]. Beyond PEP, our results demonstrating the effectiveness of rectal Tac in mitigating pancreas inflammation and damage in the classic model of cerulein-induced AP provide traction for investigations on therapeutic application of rectal Tac to protect against AP due to different etiologies.

Both rectal and oral routes for Tac delivery may be effective and safe options for PEP prophylaxis as compared to an intravenous bolus which results in toxic effects as well as adverse events, and as such clinically indicated only for a small percentage of critically ill, transplant patients. This premise is supported by our PK data showing optimal blood levels of Tac following rectal delivery or oral feeding. However, the rectal Tac may be superior to the oral Tac as the delivery through the rectum may offer several advantages such as ease of administration prior to ERCP procedure, minimal change in ERCP procedural time, by-pass of extensive pre-systemic metabolism of Tac in the GI tract which limits bioavailability (observed when Tac is given orally), by-pass of hepatic first pass elimination, and prevention of undesirable side-effects such as, nausea and vomiting. Potential bottlenecks in achieving therapeutic doses with rectal Tac may include slow and variable absorption depending on anatomical placement in the rectum (lower vs upper rectum), drug leakage following rectal placement, drug metabolism by microbiota, exclusions due to anatomical features/GI comorbidities, variable absorption due to fecal contamination and undesirable side effects. An additional limitation could be patient hesitancy in consenting to the rectal drug delivery [18,32].

The blood PK analysis of rectal Tac revealed a biphasic concentration versus time profile. This may be due to the dual nature of venous blood supply to the rectum; the upper part is linked to the portal system, whereas the lower part is directly connected with

Table 1

Comparison of PK values reported for different Tac doses and routes of administration in different species to the PK parameters observed in the current study.

Species	Route	Tac dose (mg/kg)	AUC _{0–24h} (ng•h/mL)	AUC _{0–24h} per 1 mg/kg	AUC 0–5 h or 0–6 h (ng•h/mL)	Cmax (ng/mL)	Tmax (h)	F (%) po- > IV	F (%) rectal- > po	t _{1/2} (h)	Other	Reference
Rat	oral	2	103	51.5	.	15	0.3	.	.	.	.	Sakai 2004
Rat	rectal	2	707	353.5	.	59.2	1	.	.	.	.	Sakai 2004
Mice	oral	2	.	.	95	.	.	16.9%	.	.	.	Yokogawa 1999
Rat	intra-intestinal	1	99.6	99.6	.	~100	.	25.1%	.	.	.	Okabe 2000
Rat	oral	1	10	10	.	4	0.4	5.1%	.	.	.	Zhou 2013
Healthy Human	oral	0.1	194	1940	.	34.5	1.6	.	.	24	CV% AUC 33%	Stift 2014
Healthy Human	rectal	0.2	292	1460	.	38.7	4.2	.	75.3%	27	CV% AUC 59.5%	Stift 2014
Mice	IV	1	123.3	123.3	43.8	34.6	0.15	.	.	.	.	current study
Mice	IG	1	138.9	138.9	47.5	18.84	0.21	112.7%	.	.	.	current study
Mice	rectal	1	148.7	148.7	66.2	17.93	1.625	.	107.1%	.	.	current study

Cmax = Maximum blood concentration of Tac; Tmax = Time to achieve maximum blood Tac levels; AUC = Area under the curve (measure of drug exposure after dosing); F = Bioavailability.

*Mean values are reported for the PK parameters.

the systemic blood circulation [33]. Thus, the former may yield an early spike in blood levels as it bypasses hepatic first-pass elimination, while the latter flows into the liver to achieve a later peak. PEP typically manifests 24 h post-ERCP. Our results further indicate that rectal Tac may provide a therapeutic advantage by providing adequate blood levels over an extended and required timeframe, in the desired range (lowest measured value in the experiment was 4.87 ng/mL at 24 h) to prevent PEP onset and mitigate severity, as evidenced by higher AUC values and reduction in PEP pathology. In this context, it is worth noting that the FDA recommends AUC values as the primary measure to assess the extent of drug absorption and Cmax as a secondary measure. In fact, achieving a lower effective therapeutic Tac concentration may have the added benefit of fewer toxic side-effects. Our pre-clinical data is supported by results from studies in healthy human patients where rectal Tac resulted in sustained blood levels of Tac, especially in the first 24 h post administration, compared to oral and sublingual formulations [30]. The sustained Tac levels achieved by rectal delivery may offer a potential advantage in PEP prevention, by covering a larger therapeutic window of PEP risk, which is typically thought to be 24 h, and the basis of the Cotton classification of PEP [34].

Therapeutic monitoring of Tac blood levels is important for clinical studies to determine PK, pharmacodynamics (PD), therapeutic range, dose adjustments etc. due to inter-patient variability in absorption, metabolism, and utilization of Tac. In our preclinical study, we were able to reliably quantitate blood Tac levels using ELISA over the study duration of 24 h. Future studies using complementary LC-MS/MS based approaches to measure blood, as well as target and non-target tissue (pancreas, lung, liver, spleen, and muscle) levels of Tac are warranted to estimate the systemic and local (pancreas) bioavailability of the rectal Tac formulation as compared to other systemic routes.

Systemic Tac use is often associated with adverse renal and hepatic side-effects, and we may find this to be the case with rectal Tac. While this advocates for careful patient selection, the single, low-dose rectal Tac that we used in our studies is unlikely to result in severe side effects, unlike the chronic, systemic dosing required for immune suppression in transplant subjects. As such, only mild adverse events were reported in clinical studies on use of rectal Tac in ulcerative proctitis [19,20]. Our recent studies demonstrating

safety of intraductal administration of single-dose Tac in preclinical models of PEP further support the premise that rectal Tac is unlikely to cause adverse renal or hepatotoxicity [16].

Although rectal Tac showed favorable PK values at the dose administered, there was only partial alleviation of PEP pathology. Further Tac dose optimization studies or carefully controlled, safety and efficacy testing of combination rectal Tac + indomethacin, which showed superior results than Tac alone in a large, prospective clinical study [14], are therefore warranted. In conclusion, rectal delivery of Tac shows clinical promise for PEP prophylaxis and its use in combination with indomethacin or other NSAIDs to effectively mitigate PEP can be envisioned.

Declaration of competing interest

S.Z.H is on the Scientific advisory board of ATIAS pharma.

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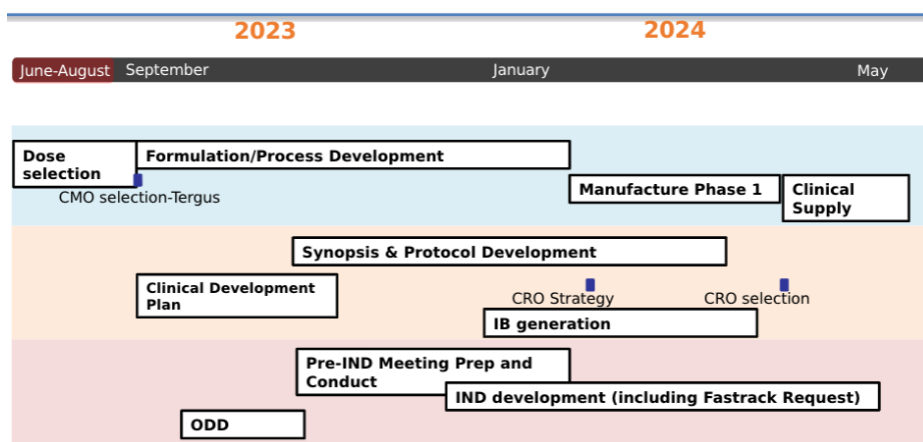
2) Additional major activities:

A. We worked with Tergus Pharma to evaluate the excipients for rectal delivery of indomethacin and tacrolimus.

No.	Excipients	Functional Category	FDA IID limit* for Topicals Rectal suppository per unit dose
1.	Glycerin	Humectant	128 mg
2.	Polyethylene Glycol 400	Solvent	0.25 mg/ mg
3.	Polyethylene Glycol 1000	Suppository base	1658 mg
4.	Polyethylene Glycol 1600	Suppository base	0.3 mg/ mg
5.	Polyethylene Glycol 3350	Suppository base	1425.96 mg
6.	Polyethylene Glycol 4000	Suppository base	1269 mg
7.	Polyethylene Glycol 6000	Suppository base	128 mg
8.	Polyethylene Glycol 8000	Suppository base	52 mg
9.	Benzyl alcohol	Solvent, solubilizer	129 mg
10.	Hard fat	Suppository base	2000 mg
11.	Polysorbate 80	Surfactant	72.15 mg
12.	Vegetable oil, hydrogenated	Suppository base	1964.99 mg
13.	Vegetable oil Glyceride, hydrogenated	Suppository base	870 mg
14.	White wax	Suppository base	265 mg
15.	Cocoa butter	Suppository base	2280 mg
16.	Purified water	Solvent	-
17.	#Propylene carbonate	Solvent	5.0% w/w
18.	#Propylene glycol	Solvent	20.0% w/w
19.	#Hydrogenated castor oil	Suppository base	2.0% w/w

B. We worked with Premier Research to develop a strategic plan for IND application.

Timeline



C. We worked with Medicilon Inc. to evaluate preclinical pharmacokinetic profiles of a novel diclofenac (Dic) + tacrolimus (Tac) formulations.

Study Design

Experimental Group	Route	Dose level mg/kg	Dose conc mg/ml (mix of Dic+Tac)	Dose volume mL/kg	No. of doses	Samples	Analytes measured
1	Rectal	Dic-20.8 Tac- 1.04	Dic- 26 Tac- 1.3	0.8 mL/kg (0.02 mL/ 25g mouse)	single	Whole blood, plasma and tissue	Dic and Tac (LC-MS/MS)
2	IV	Dic-20.8 Tac- 1.04	Dic-5.2 Tac-0.26	4 mL/kg (0.1 mL/25 g mouse)	single	Whole blood, plasma and tissue	Dic and Tac
3	PO (gavage)	Dic-20.8 Tac- 1.04	Dic- 5.2 Tac-0.26	4 mL/kg (0.1 mL/25 g mouse)	single	Whole blood, plasma and tissue	Dic and Tac