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TITLE: Mucin-Based Biotherapies for *Pseudomonas aeruginosa* Lung Infection

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this project is to demonstrate that MUC1 synthetic peptides will protect against Pseudomonas aeruginosa lung infection using both in vitro and in vivo model systems. For months 1-12 of the project, studies were conducted demonstrating that MUC1 20-,40-, 60-, 80-, and 100-mer peptides 1) bound to P. aeruginosa bacteria and its flagella, 2) competitively inhibited P. aeruginosa and its flagella binding to human lung cells, and flagella-dependent bacterial motility 3) were not cytotoxic to lung cells, 4) did not affect lung cell barrier formation, 5) exhibited no damage to mouse lung, liver or kidney when administered in vivo, and 6) displayed appropriate lung bioavailability in vivo. P. aeruginosa laboratory and clinical strains were tested. In all of the assays performed, the MUC1 peptides exhibited a relative potency according to the rank order 100-mer > 80-mer > 60-mer > 40-mer > 20-mer. These studies, and those to be performed in months 13-18 of the project, will provide preclinical data for future human clinical trials.

15. SUBJECT TERMS
mucin-1; MUC1; synthetic peptide; Pseudomonas aeruginosa; flagella; lung; infection; antibiotic; epithelial; adhesion, motility

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	12	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION: More than 250,000 *Pseudomonas aeruginosa* infections are estimated to occur annually in the U.S. with an associated healthcare cost greater than $2 billion. The widespread use of antimicrobial antibiotics has led to the emergence of difficult-to-treat, multidrug-resistant *Pseudomonas aeruginosa* strains, necessitating the use of less-desirable, alternative drugs that are severely limited by their decreased effectiveness and increased toxicity and costs. Therefore, alternative therapies to treat *Pseudomonas aeruginosa* infections are urgently needed. Based on previous research, we propose that synthetic peptide drugs derived from a naturally-produced lung protein, MUC1, will offer new treatment options for patients with *Pseudomonas aeruginosa* lung infections. MUC1 peptide drugs will provide a novel class of *Pseudomonas aeruginosa* therapeutics with reduced off-target effects and enhanced effectiveness in the clinic. Because the mode of action of MUC1 peptide drugs in preventing *Pseudomonas aeruginosa* infection relies on inhibiting bacterial adhesion to human lung cells, rather than a direct bactericidal approach as with current antibiotics, MUC1 peptides are unlikely to spur the development of bacterial resistance. In this project, MUC1 peptides will be tested for their ability to inhibit *Pseudomonas aeruginosa* lung infection using both in vitro and in vivo techniques. We expect to identify at least 1 peptide with a high therapeutic index for treating an ongoing *Pseudomonas aeruginosa* infection, and that is readily bioavailable and nontoxic following aerosol delivery to mouse lungs in an experimental model of *Pseudomonas aeruginosa* pneumonia. We envision that, once commercialized, MUC1 peptide therapeutics will be used in combination with current standard of care antibiotics to increase patient survival beyond that achieved by antibiotics alone.

2. Keywords: mucin-1; MUC1; synthetic peptide; *Pseudomonas aeruginosa*; flagella; lung; infection; antibiotic; epithelial; adhesion, motility

3. ACCOMPLISHMENTS

3A. Major goals of the project (months 1-12)

3A1. Major Task 1. Determine MUC1-ED synthetic peptide binding to *P. aeruginosa* and its flagella.

3A1A. Subtask 1. Obtain all necessary materials and supplies, including MUC1-ED synthetic peptides, *P. aeruginosa* strains, and human airway epithelial cells (month 1).

3A1B. Subtask 2. Test *P. aeruginosa* strains on motility agar (month 1).

3A1C. Subtask 3. Purify *P. aeruginosa* flagellins and cross-link to agarose (month 2).

3A1D. Subtask 4. Measure binding affinity of Alexa Fluor 488-labeled MUC1-ED peptides for *P. aeruginosa* and flagellin-agarose using fluorescence polarization assays (months 2-4).

3A2. Major Task 2. MUC1-ED peptide inhibition of *P. aeruginosa* and flagella binding to airway epithelial cells.

3A2A. Subtask 1. Perform competitive binding assays with MUC1-ED peptides for *P. aeruginosa* and flagellin binding to human airway epithelial cells (months 5-7).

3A2B. Subtask 2. Perform bacterial motility assays with MUC1 peptides. (months 8-9).

3A3. Major Task 3. Test Effects of MUC1-ED peptides on airway epithelial cell toxicity and monolayer integrity.

3A3A. Subtask 1. Test MUC1-ED peptides for airway epithelial cell toxicity and monolayer integrity, and measure therapeutic index for the optimum MUC1-ED peptides (months 10-12).

3A4A. Major Task 4A (optional). If necessary, perform alternative protocols to resolve potential pitfalls with MUC1-ED synthetic peptides.

3A4A1. Subtask 1. MUC1-ED gene segments will be synthesized corresponding to 20-, 40-, 60-, 80-, and 100-amino acid regions of the VNTR, codon-optimized for expression in *E. coli*, and the corresponding recombinant MUC1-ED proteins purified (month 12). Major Task 4A, Subtask 1 was not performed

3A4B. Major Task 4B. Obtain IACUC approval.

3A5B1. Subtask 1. Obtain USAMRMC ORP and local IACUC approval (months 9-12).

3A5. Major Task 5. Purchase mice.

3A5A. Purchase C57BL/6 mice from a certified commercial vendor (n=100) (month 12).

3A6. Major Task 6. *P. aeruginosa* pretreatment with MUC1-ED peptides prior to infection.

3A6A. Subtask 1. Perform dose tolerance and pharmacokinetic studies of MUC1-ED peptides in mice (month 12).

3B. Accomplishments under these goals (months 1-12)

3B1. Major Task 1. Determine MUC1-ED synthetic peptide binding to *P. aeruginosa* and its flagella.

3B1A. Subtask 1. Obtain all necessary materials and supplies, including MUC1-ED synthetic peptides, *P. aeruginosa* strains, and human airway epithelial cells (month 1). Materials and supplies were purchased from commercial vendors or obtained from in-house sources.

Table 1. Materials and supplies

Item	Source
Blood agar plates	VWR
TSA agar plates	VWR
Ethanol, 4x4L	Sigma-Aldrich
PNA-agarose	Vector Labs
BSA, 30%	VWR
Crystal violet	Sigma-Aldrich
Acetic acid	Sigma-Aldrich
MUC1-ED Ab	LSBio
Tissue homogenizer	VWR
APS, TEMED	Sigma-Aldrich
Protein A/G agarose	Invitrogen
P. aeruginosa, PAK	In-house
P. aeruginosa, PA01	In-house
P. aeruginosa, clinical #1	In-house
P. aeruginosa, clinical #2	In-house
P. aeruginosa, clinical #3	In-house
P. aeruginosa, clinical #4	In-house
P. aeruginosa, clinical #5 (MDR)	In-house
MUC1-ED synthetic peptide, 20-mer	In-house
MUC1-ED synthetic peptide, 40-mer	In-house
MUC1-ED synthetic peptide, 60-mer	In-house
MUC1-ED synthetic peptide, 80-mer	In-house
MUC1-ED synthetic peptide, 100-mer	In-house
Normal human bronchial epithelial (NHBE) cells	In-house

3B1B. Subtask 2. Test *P. aeruginosa* strains on motility agar (month 1). *P. aeruginosa* laboratory strains (PAK, PAO1, PAK/ΔfliC), and isolates from patients with lung infections (clinical strains 50241, 50255, 50296, 50312, 50327), including multidrug-resistant (MDR) bacteria, were washed and resuspended in Luria-Bertani (LB) culture broth. The bacteria were stab-inoculated into 0.3% LB agar plates, incubated overnight, and colony diameters (length, width) measured as an indicator of bacterial motility. All bacteria exhibited motility, with the exception of the flagella-deficient PAK/ΔfliC negative control (Table 2).

Table 2. *Pseudomonas aeruginosa* motility

P. aeruginosa	Diameter 1 (mm)	Diameter 2 (mm)	Average
PAK	11.0	11.5	11.3

PA01	14.5	14.5	14.5
Clinical strain 50241	12.0	13.0	12.5
Clinical strain 50255	10.5	10.0	10.3
Clinical strain 50296	11.5	15.5	13.5
Clinical strain 50312	12.5	12.5	12.5
Clinical strain 50327	10.5	10.0	10.3
PAK/ΔfliC	0.0	0.0	0.0

3B1C. Subtask 3. Purify *P. aeruginosa* flagellins and cross-link to agarose (month 2). An overnight culture of *P. aeruginosa* strains PA01, PAK, and the flagellin-deficient PAK/ΔfliC were centrifuged at 5,000xg for 30 min, resuspended in Krebs-Ringer buffer, and incubated for 1 h at 37°C. The bacteria were collected by centrifugation, and the flagella-containing supernatant filtered through a 0.22-µm pore membrane and the filtrate boiled for 20 min. The filtrate was concentrated by centrifugal ultrafiltration, adjusted to pH 6.0, and flagellin proteins were purified from the isolated flagella by sequential ion exchange chromatography using Macro-Prep High S and Macro-Prep HighQ supports. Aliquots of column fractions were resolved by SDS-PAGE and stained with Coomassie Blue to detect the 40-50 kiloDalton (kDa) flagellin protein bands (Figure 1). Other column aliquots were processed for flagellin immunoblotting and for *P. aeruginosa* pilin immunoblotting to confirm the absence of pilin contamination (Figure 2). Flagellin-containing fractions were incubated with polymyxin B-agarose to remove endotoxin, after which less than 0.1 endotoxin unit/µg of protein was detected by the *Limulus* amebocyte lysate assay. For flagellin chemical cross-linking to agarose, solutions of 1.0 mg/ml of the a-type and b-type flagellins were added to NHS-activated agarose in a spin column and mixed with end-over-end rotation for 1 h at 22°C. The agarose was collected by centrifugation at 1,000xg for 1 min , and washed once with PBS, pH 7.4. The agarose was incubated with 1.0 M Tris, pH 8.0 for 1 h at 22°C to block unreacted sites, washed twice with PBS, pH 7.4, and stored at 4°C until use.

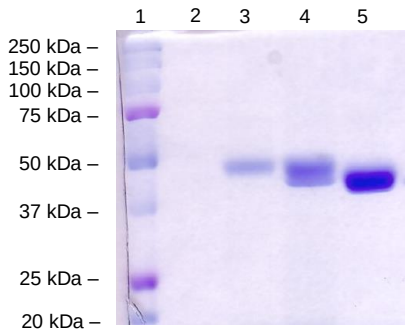

Figure 1. Purified flagellins from *P. aeruginosa*. Lane 1, prestained molecular weight marker proteins. Lane 2, flagellin-deficient strain PAK/ΔfliC. Lane 3, a-type flagellin strain PAK. Lane 4, b-type flagellin strain PA01. Coomassie blue stain.

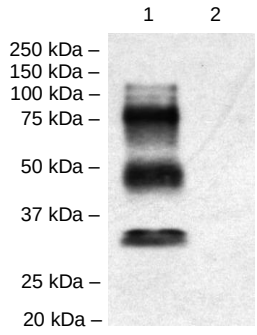

Figure 2. Pilin immunoblot to confirm the absence of pilin contamination. Lane 1, positive pilin control. Lane 2, a-type flagellin purified from strain PAK.

3B1D. Subtask 4. Measure binding affinity of Alexa Fluor 488-labeled MUC1-ED peptides for *P. aeruginosa* and flagellin-agarose using fluorescence polarization assays (months 2-4). The 20-mer monomer MUC1-ectodomain (MUC1-ED) synthetic peptide (GSTAPPAHGVTSAPDTRPAP) and head-to-tail 40-mer, 60-mer, 80-mer, and 100-mer peptides of the 20-mer were labeled with Alexa Fluor 488. $1x10^6$ colony forming units (CFUs)/ml of *P. aeruginosa* strains PAK or PA01, or an equivalent amount of a-type or b-type flagella-agarose, were incubated for 1 hr at 4°C with increasing concentrations of labeled peptides (0, 0.5, 1.0, 2.5, 5.0, 10, 20, 20 ng/well). Unbound peptide was removed by centrifugation at

5,000xg for 10 min at 4°C and the remaining bound fluorescence measured at 488 nm. Fluorescence polarization (Figure 3) and Scatchard analysis (Figure 4) of the binding data was performed, and the dissociation constant (Kd) of each peptide-flagellin interaction was determined in nM. The results demonstrated that the 20-mer, 40-mer, 60-mer, 80-mer, and 100-mer peptides bound to both the PAK and PA01 bacterial strains, and to both a-type and b-type flagella. Scatchard analysis of the peptide binding demonstrated that the peptides bound to both *Pseudomonas aeruginosa* and its flagella with the rank order 100-mer > 80-mer > 60-mer > 40-mer > 20-mer.

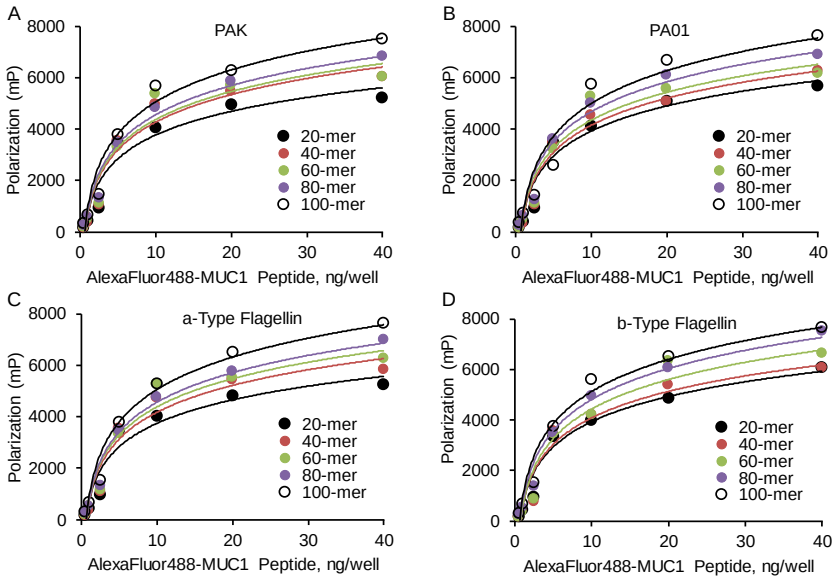

Figure 3. Fluorescence polarization analysis of AlexaFluor488 MUC1 peptides binding to (A) PAK bacteria, (B) PA01 Bacteria, (C) a-type flagellin, and (D) b-type flagellin.

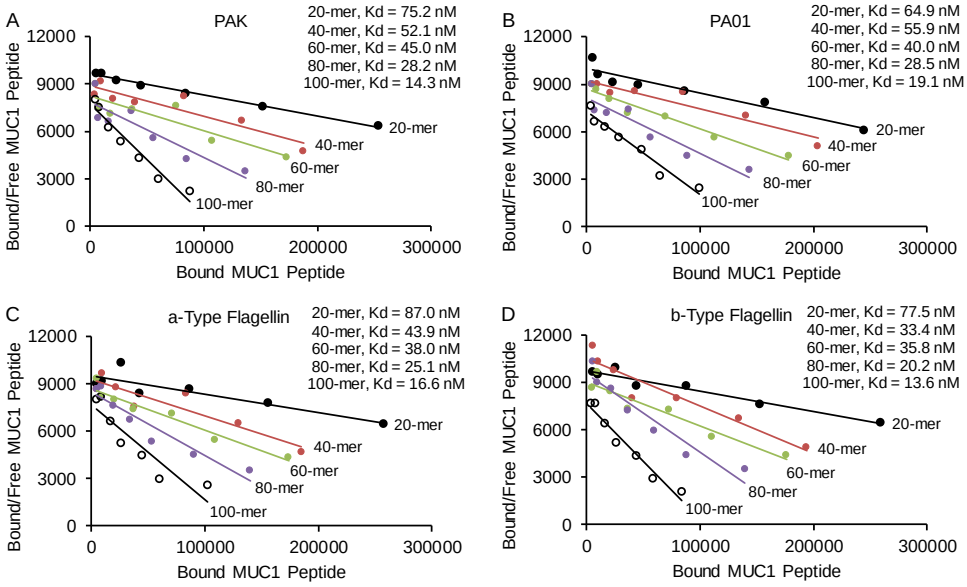

Figure 4. Scatchard analysis of AlexaFluor488 MUC1 peptides binding to (A) PAK bacteria, (B) PA01 Bacteria, (C) a-type flagellin, and (D) b-type flagellin. The affinity (Kd) for each binding interaction is indicated in the upper right of each panel.

3B2. Major Task 2. MUC1-ED peptide inhibition of *P. aeruginosa* and flagella binding to airway epithelial cells.

3B2A. <u>Subtask 1. Perform competitive binding assays with MUC1-ED peptides for *P. aeruginosa* and flagellin binding to human airway epithelial cells (months 5-7).</u>

NHBE cells in 24-well plates ($2x10^5$ cells/well) were incubated with *P. aeruginosa* laboratory strains, PAK and PA01, and clinical strain, 50241 ($2x10^7$ CFUs/well) in the presence of increasing concentrations of MUC1-ED peptides or scrambled control peptides. The cells were washed and bacterial CFUs bound to the cells quantified on agar plates (Figure 5). Binding of Alexa Fluor 488-labeled a-type and b-type flagella to NHBE cells will be studied in the presence of increasing concentrations of MUC1-ED peptides or scrambled controls (Figure 6). Half-maximal inhibitory concentration (IC_{50}) values for the peptides were calculated based on the binding inhibition curves. The results demonstrated that all of the peptides dose-dependently inhibited *P. aeruginosa* and flagella binding to the cells with the rank order 100-mer > 80-mer > 60-mer > 40-mer > 20-mer.

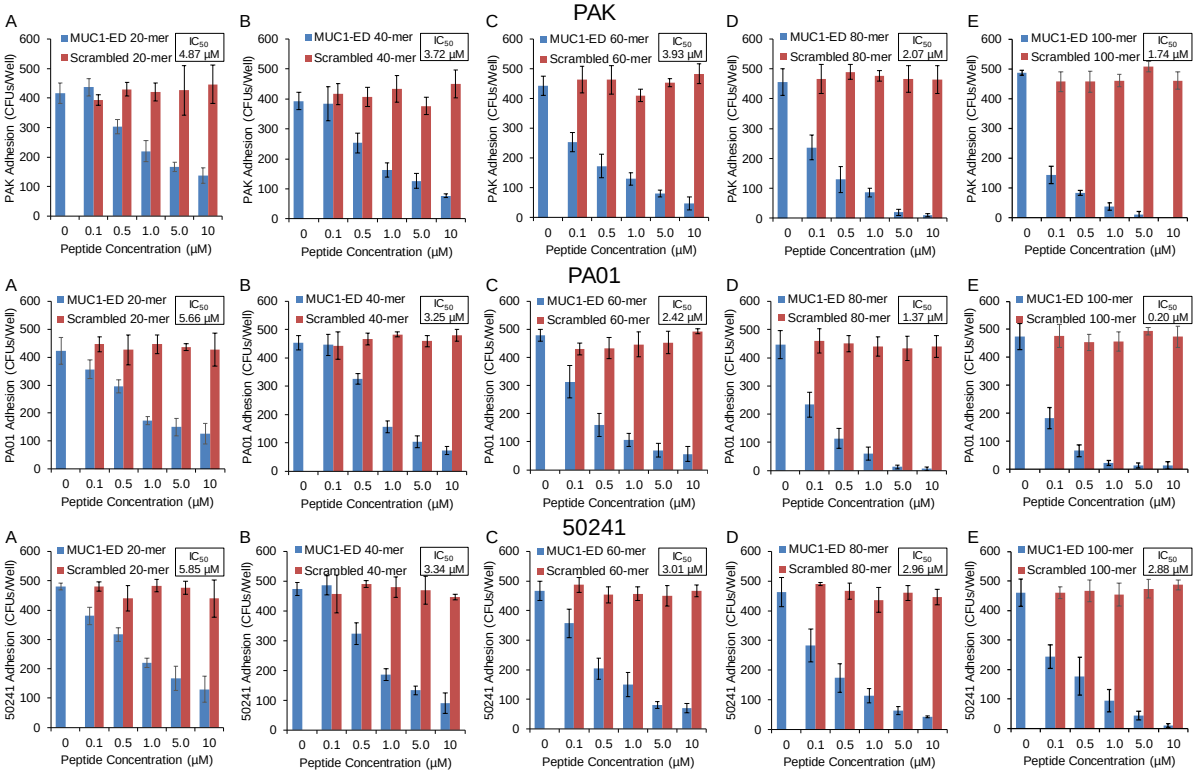

Figure 5. Inhibition of binding of *P. aeruginosa* strains PAK (upper), PA01 (middle), and 50241 (lower) to NHBE cells by (A) 20-mer, (B) 40-mer, (C) 60-mer, (D) 80-mer, and (E) 100-mer MUC1 peptides, or the corresponding scrambled control peptides. The IC_{50} value for each MUC1 peptide inhibition is indicated in the upper right of each panel.

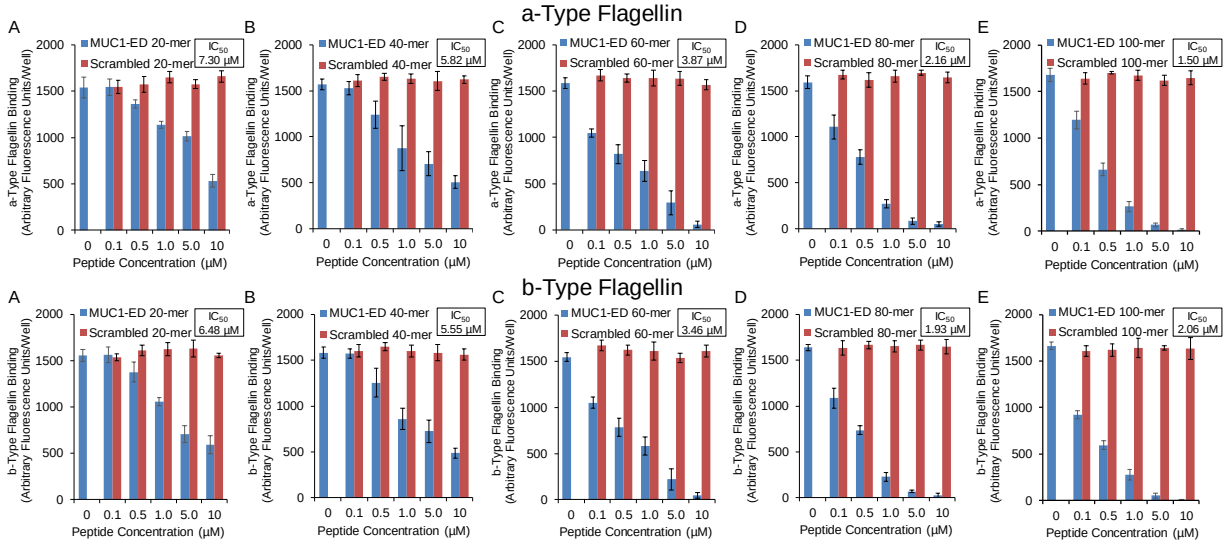

Figure 6. Inhibition of binding of *P. aeruginosa* a-type flagellin (upper) and b-type flagellin (lower) to NHBE cells by (A) 20-mer, (B) 40-mer, (C) 60-mer, (D) 80-mer, and (E) 100-mer MUC1 peptides, or the corresponding scrambled control peptides. The IC_{50} value for each MUC1 peptide inhibition is indicated in the upper right of each panel.

3B2B. Subtask 2. Perform bacterial motility assays with MUC1 peptides. (months 8-9). Increasing concentrations of the MUC1 peptides were incorporated into 0.3% motility agar. *P. aeruginosa* laboratory strains PAK and PA01, and clinical strain 50241 were stab-inoculated into the agar, incubated overnight, and colony diameters measured as an indicator of bacterial motility. As shown in Figure 7, the peptides dose-dependently inhibited motility of all bacterial strains with the rank order 100-mer > 80-mer > 60-mer > 40-mer > 20-mer. As a negative control, the flagella-deficient PAK/ΔfliC strain did not exhibit bacterial motility (Table 2).

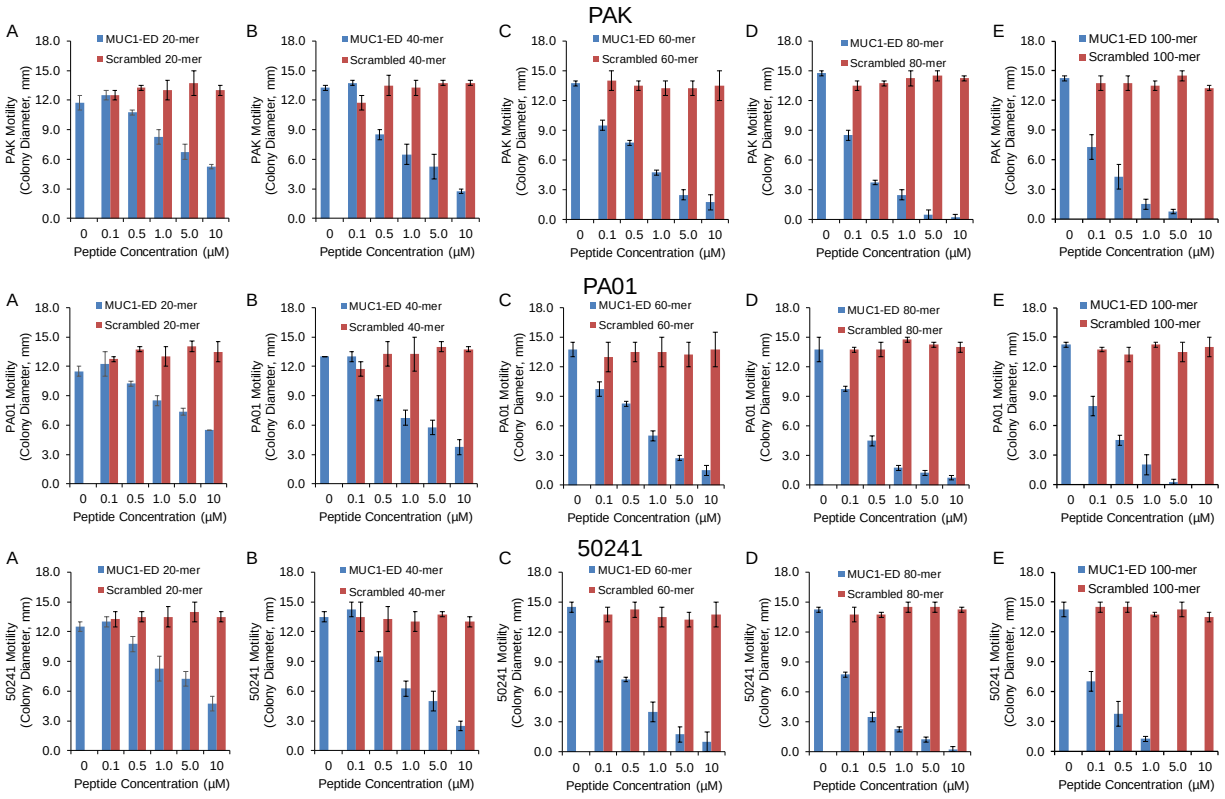

Figure 7. Inhibition of motility of *P. aeruginosa* strains PAK (upper), PA01 (middle), and 50241 (lower) by (A) 20-mer, (B) 40-mer, (C) 60-mer, (D) 80-mer, and (E) 100-mer MUC1 peptides, or the corresponding scrambled control peptides.

3B3. Major Task 3. Test Effects of MUC1-ED peptides on airway epithelial cell toxicity and monolayer integrity.

3B3A. Subtask 1. Test MUC1-ED peptides for airway epithelial cell toxicity and monolayer integrity, and measure therapeutic index for the optimum MUC1-ED peptides (months 10-12). Starting at 5.0 mM, $\log_{10}$ dilutions of each peptide were incubated with NHBE cells for 24 hr and viability determined using 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium. To assess the effects of MUC1 peptides on monolayer integrity, a nontoxic dose of peptides was added to the apical surface of NHBE cells and transepithelial electrical resistance (TEER) measured. None of the peptides at any tested concentrations affected cell viability (Figure 8A) or monolayer integrity (Figure 8B).

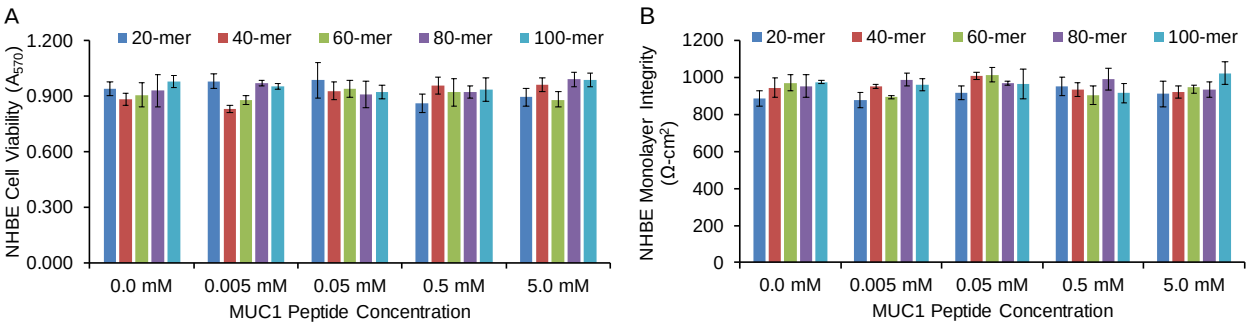

Figure 8. Effect of MUC1 peptides on NHBE (A) cell viability and (B) monolayer integrity.

3B4A. Major Task 4A (optional). If necessary, perform alternative protocols to resolve potential pitfalls with MUC1-ED synthetic peptides.

3B4A1. Subtask 1. MUC1-ED gene segments will be synthesized corresponding to 20-, 40-, 60-, 80-, and 100-amino acid regions of the VNTR, codon-optimized for expression in *E. coli*, and the corresponding recombinant MUC1-ED proteins purified (month 12). This Subtask was not performed since it was only proposed as a backup in the event that the 40-mer, 60-mer, 80-mer, and 100-mer peptides did not exhibit increased potency compared with the 20-mer. However, as shown in Figures 3-7, the MUC1 peptides exhibited a rank-order profile of potency of 100-mer > 80-mer > 60-mer > 40-mer > 20-mer.

3B4B. Major Task 4B. Obtain IACUC approval.

3A5B1. Subtask 1. Obtain USAMRMC ORP and local IACUC approval (months 9-12). USAMRMC ORP approval was obtained on 12 February 2019. University of Maryland Baltimore IACUC approval was obtained on 15 June 2018.

3B5. Major Task 5. Purchase mice.

3A5A. Subtask 1. Purchase C57BL/6 mice from a certified commercial vendor (n=100) (month 12). Mice were purchased from Jackson Labs and passed quarantine on 20 November 2019.

3B6. Major Task 6. *P. aeruginosa* pretreatment with MUC1-ED peptides prior to infection.

3A6A. Subtask 1. Perform dose tolerance and pharmacokinetic studies of MUC1-ED peptides in mice (month 12). Mice were administered intranasally with 5.0 mM of the MUC1 80-mer and 100-mer peptides, the most efficient peptides for inhibiting *P. aeruginosa* adhesion/flagella binding established in Major Tasks 1-3. At 24 hr post-administration, the mice were sacrificed and lungs, liver, and kidneys harvested and assessed for histological signs of damage by H&E staining. Neither of the peptides exhibited signs of organ damage (Figure 9). MUC1 and scrambled control peptide levels in lung homogenates were measured at 0, 1, 2, 4, 8, and 24 hr post-administration by enzyme-linked immunosorbent assay (ELISA) to assess lung pharmacokinetics and peptide half-life ($t_{1/2}$) values calculated. The results indicated that all peptides exhibited equivalent $t_{1/2}$ values (Figure 10).

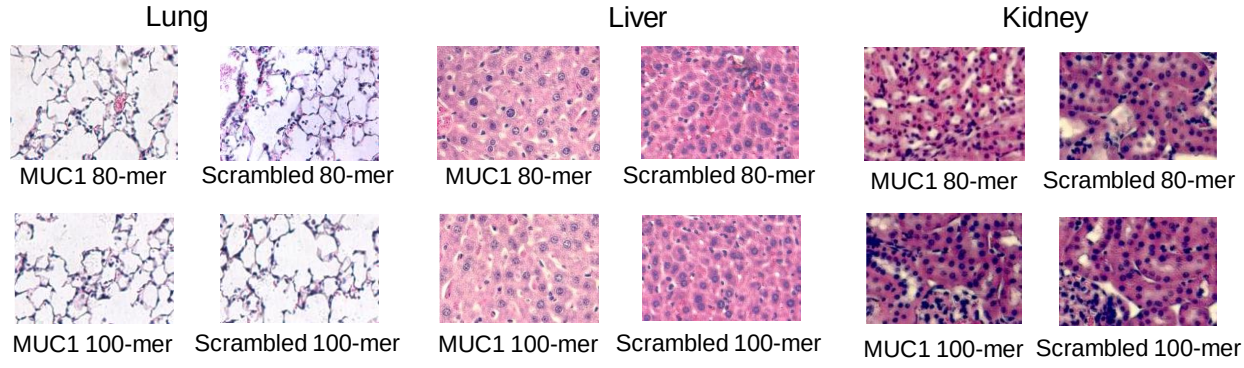

Figure 9. Dose tolerance effect of MUC1 80-mer and 100-mer peptides on mouse organ damage as assessed by H&E staining.

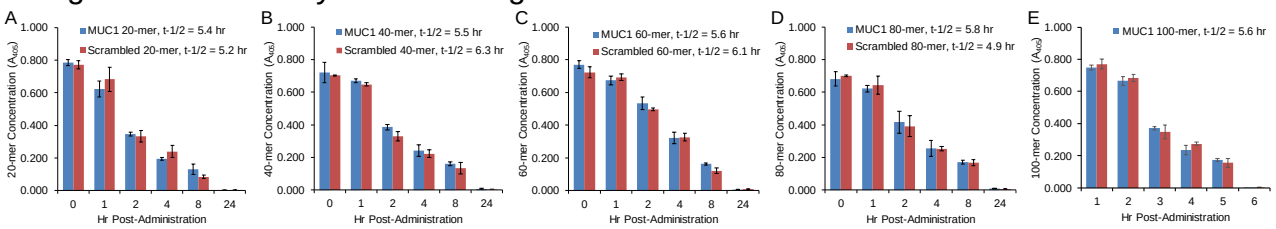

Figure 10. Lung pharmacokinetics of (A) 20-mer, (B) 40-mer, (C) 60-mer, (D) 80-mer, and (E) 100-mer MUC1 and scrambled control peptides. The $t_{1/2}$ values for each peptide are indicated in the upper right of each panel.

3C. Opportunities for training and professional development: Nothing to Report
3D. Dissemination of results to communities of interest: Nothing to Report
3E. Plans for the next reporting period to accomplish the goals: For the next reporting period, we plan to continue the studies as outlined in the Statement of Work for months 13-18.

4. IMPACT

4A. Impact on development of the principal discipline(s) of the project: Nothing to Report
4B. Impact on other disciplines: Nothing to Report
4C. Impact on technology transfer: Nothing to Report
4D. Impact on society beyond science and technology: Nothing to Report

5. CHANGES/PROBLEMS: Nothing to Report

6. PRODUCTS

6A. Publications, conference papers, and presentations: Nothing to Report
6B. Website(s) or other Internet site(s): Nothing to Report
6C. Technologies or techniques: Nothing to Report
6D. Inventions, patent applications, and/or licenses: Nothing to Report
6E. Other products: Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7A. Individuals who have worked on the project:

Name	Erik P. Lillehoj, PhD
Project Role	PI
Research Identifier	ORCID: 0000-0003-0910-6074
Nearest person month worked	6
Contribution to Project	Dr. Lillehoj performed the studies, analyzed the results and prepared data graphs and tables.
Funding Support	Nothing to Report

7B. Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period: Nothing to Report

7C. Other organizations were involved as partners: Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS: Nothing to Report

9. APPENDICES: Nothing to Report