AWARD NUMBER: W81XWH-16-1-0284

TITLE: Development of New Therapeutics Targeting Biofilm Formation by the Opportunistic Pulmonary Pathogens Pseudomonas aeruginosa and Aspergillus Fumigatus

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REPORT DATE: MAY 2020

TYPE OF REPORT: Annual Technical Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
MAY 2020	Annual Technical Report	15 Sep 2019 – 14 APR 2020

4. TITLE AND SUBTITLE

Development of New Therapeutics Targeting Biofilm Formation by the Opportunistic Pulmonary Pathogens *Pseudomonas aeruginosa* and *Aspergillus fumigatus*

5a. CONTRACT NUMBER
W81XWH-16-1-0284

5b. GRANT NUMBER
PR150786-P1

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Major accomplishments this period:

1. BCE5582, a *Bacillus cereus* orthologue of PelA, previously demonstrated a similar efficiency for biofilm disruption than PelA, a higher resistance to elastase and a longer half-life in mouse lung (Major Task 4). Study of that GH variant was therefore continued during this report period. Tolerability and efficacy of BCE5582 was comparable to PelA in a neutrophil-depleted model of acute invasive aspergillosis. During the next report period, pharmacokinetics and *in vivo* efficacy assays will be replicated.

2. *In vitro* experiments identified the combinations posaconazole/Sph3 and caspofungin/PelA as the most effective against *A. fumigatus* (Major Task 1). In our neutropenic model of acute aspergillosis, Sph3 potentiated the effect of posaconazole, as measured through the pulmonary fungal burden (Major Task 7). The ability of PelA and Sph3 to potentiate caspofungin are now being investigated. During this report period, we determined the optimal dose of caspofungin for potentiation studies in this murine model. During next report period, we will perform the GH + caspofungin combination studies *in vivo*.

3. Ciprofloxacin and ceftazidime in combination with PslG/PelA and PslG/Ega3 exhibited the greatest antibacterial activity in our *in vitro* assays (Major Task 1). In a model of acute murine *P. aeruginosa* infection, PslG/PelA but not PslG/Ega3 enhanced the activity of ciprofloxacin (previous period, Major Task 7). Studies during this report period demonstrated that neither PslG/PelA nor PslG/Ega3 were able to enhance the activity of ceftazidime (Major Task 7) in this model of acute infection. In a chronic model of *P. aeruginosa* pulmonary infection, no potentiation of ciprofloxacin was observed with either PslG/PelA or PslG/Ega3.

15. SUBJECT TERMS
Pseudomonas aeruginosa; *Aspergillus fumigatus*; virulence; biofilm; exopolysaccharide; glycoside hydrolase; antimicrobial potentiation.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	23	**19b. TELEPHONE NUMBER**
Unclassified	Unclassified	Unclassified			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION.

The bacteria *Pseudomonas aeruginosa* (PA) and fungus *Aspergillus fumigatus* (AF) are common causes of pulmonary disease in immunocompromised patients. These infections are associated with high morbidity and mortality, underscoring the urgent need for new effective therapies for these conditions. During pulmonary infection, both pathogens form biofilms, which enhance resistance to antimicrobials and immune defenses. Biofilm formation is dependent on the synthesis of matrix exopolysaccharides – Pel, Psl for PA, galactosaminogalactan (GAG) for AF. Exopolysaccharide-deficient mutants of PA and AF are less virulent in animal models, suggesting that these glycans are promising therapeutic targets. We have identified and produced recombinant versions of microbial glycoside hydrolase (GH) enzymes, PelA, and PslG from PA and Ega3, and Sph3 from AF, which degrade exopolysaccharides and disrupt biofilms *in vitro*. We hypothesize that treatment with these GHs alone or in combination with antimicrobials will be well tolerated and improve outcomes in experimental pulmonary infection with PA and AF. We therefore propose the following studies: (1) To characterize the ability of recombinant GH enzymes to enhance the activity of antimicrobial agents against PA and AF *in vitro* (2) Perform tolerability and pharmacokinetic studies of intratracheal therapy with recombinant GH in mice. (3) Evaluate the efficacy of GH therapy alone and in combination with antimicrobials for the treatment of acute and chronic PA and AF infection mouse models. In the short term these studies will provide solid preliminary data for the preclinical evaluation of pulmonary GH therapy against two of the most important opportunistic pulmonary pathogens. In the long-term, these results can also be extended to develop GH therapy pulmonary infections with other exopolysaccharide-producing pathogens such as *Staphylococcus*, *Acinetobacter* and *Mucor* species.

KEYWORDS.

Pseudomonas aeruginosa; *Aspergillus fumigatus*; virulence; biofilm; exopolysaccharide; glycoside hydrolase; antimicrobial potentiation.

2. ACCOMPLISHMENTS:

What were the major goals of the project?

Please note this is <u>partnered award</u> with research being performed at McGill University (PI: Sheppard) and The Hospital for Sick Children (PI: Howell). The material presented herein pertains to both awards. Award numbers: W81XWH-16-1-0283 and W81XWH-16-1-0284
Please note also that this Annual report covers an 8 month period, from September 15th, 2019, to May 14th, 2020.

<u>MAJOR GOALS FOR YEAR 4:</u>

SPECIFIC AIM 1: TO CHARACTERIZE THE ABILITY OF THE HYDROLASES TO ENHANCE THE ACTIVITY OF ANTIMICROBIAL AGENTS *IN VITRO*.

<u>Major Task 1</u>: Identify antimicrobials that are potentiated in the presence of candidate hydrolases. ***Achieved Year 2.***

Milestone achieved: Identification of hydrolase-antimicrobial combinations that synergize against A. fumigatus *and* P. aeruginosa*. These antimicrobials will be prioritized and used in Aim3.*

SPECIFIC AIM 2: TO PERFORM PRELIMINARY TOLERABILITY AND PHARMACOKINETIC STUDIES OF CANDIDATE HYDROLASES *IN VIVO*.

<u>Major Task 2</u>: Test candidate hydrolases for toxicity *in vivo*. ***Achieved Year 2.***

Milestone achieved: Obtain Animal use approval.

Milestone achieved: Evaluation of pulmonary toxicity of candidate hydrolase regimens.

<u>Major Task 3</u>: Pharmacokinetic studies of candidate hydrolases. ***Achieved Year 3.***

Milestone achieved: Evaluation of pharmacokinetics of candidate hydrolase regimens.

<u>Major Task 4</u> (as required): Development of candidate hydrolase variants

Subtask 1: Express and purify Sph3, Ega3, PelA and PslG for subtasks 2 – 5. (Months 9-15) (PI: Howell) ***Achieved Year 1.***

Subtask 2: Test protease resistance of candidate hydrolases against *A. fumigatus* isolates in the epithelial cell damage assay using western blot analysis, and mass spectrometry (as required). (Months 9-15) (PI: Sheppard-Howell)

Subtask 3: Test protease resistance of candidate hydrolases against *P. aeruginosa* isolates in the epithelial cell damage assay using western blot analysis, and mass spectrometry (as required). (Months 9-15) (PI: Howell)

Subtask 4: Test chemical modification as a means to increase the stability of candidate hydrolases (as required). (Months 9-21) (PI: Sheppard-Howell)

Subtask 5: Test site-specific modification as a means to increase the stability of candidate hydrolases (as required). (Months 9-21) (PI: Sheppard-Howell) ***Achieved Year 2.***

Milestone: Development of stable candidate hydrolases (Month 21).

SPECIFIC AIM 3: TO EVALUATE CANDIDATE HYDROLASES ALONE AND IN COMBINATION WITH ANTIMICROBIAL AGENTS IN THE TREATMENT OF EXPERIMENTAL *A. FUMIGATUS* AND *P. AERUGINOSA* PULMONARY INFECTIONS *IN VIVO*.

Major Task 5: Test hydrolases for activity in animal models of acute disease. ***Achieved Year 3.***

Milestone achieved: Determine efficacy of candidate hydrolase regimens in the treatment of acute infection with A. fumigatus *and* P. aeruginosa *(Month 30).*

Major Task 6: Test hydrolases for activity in animal models of chronic disease

Subtask 1: Express and purify Ega3, PelA and PslG for subtasks 2 – 3 (Months 13-30) (PI: Howell) ***Achieved Year 1.***

Subtask 2: Determine the effects of candidate hydrolases (Ega3) on fungal burden of immunocompetent mice chronically infected with *A. fumigatus.* (Months 13-30) (PI: Sheppard) ***Achieved Year 3.***

Subtask 3: Determine the effects of candidate hydrolases (PslG/PelA and PslG/Ega3 combinations) on bacterial burden of immunocompetent mice chronically infected with *P. aeruginosa*. (Months 18-30) (PI: Sheppard)

Milestone: Determine efficacy of candidate hydrolase regimens in the treatment of chronic infection with A. fumigatus *and* P. aeruginosa. *(Month 30)*

Major Task 7: Test hydrolases for synergy with antimicrobials

Subtask 1: Express and purify Sph3, Ega3, PelA and PslG for subtasks 2 – 3. (Months 25-36) (PI: Howell) ***Achieved Year 1.***

Subtask 2: Determine the effects of hydrolase (Sph3, Ega3, PelA)-antifungal combinations on fungal burden of mice infected with *A. fumigatus*. (Months 25-36) (PI: Sheppard) ***Achieved Year 3***

Subtask 3: Determine the effects of hydrolase (PslG/PelA and PslG/Ega3)-antibiotic combinations on burden of mice infected with *P. aeruginosa*. (Months 25-36) (PI: Sheppard)

Milestone: Show a proof-of-concept for candidate hydrolases for use in treatment of A. fumigatus *and* P. aeruginosa*. Get ready to initiate trials of delivery systems and detailed pharmacodynamics experiments as a prelude to Phase I clinical trials. (Month 36)*

What was accomplished under these goals?

Accomplishments for Year 4:

Routine production of recombinant GHs in our labs has become efficient enough to meet our needs in quantity and quality for all experiments under this DoD grant**: Subtask 1, for Major Tasks 1 to 7: completed.**

SPECIFIC AIM 1: TO CHARACTERIZE THE ABILITY OF THE HYDROLASES TO ENHANCE THE ACTIVITY OF ANTIMICROBIAL AGENTS *IN VITRO.*

MAJOR TASK 1: Identify antimicrobials that are potentiated in the presence of candidate glycoside hydrolases (GH).

☑ **Milestone Achieved: Identification of hydrolase-antimicrobial combinations that synergize against *A. fumigatus* and *P. aeruginosa*.** Most effective GH/antifungal combinations were posaconazole/Sph3 and caspofungin/PelA. Most effective GH/antibacterial combinations were ciprofloxacin and ceftazidime with PslG/PelA and PslG/Ega3.

SPECIFIC AIM 2: TO PERFORM PRELIMINARY TOLERABILITY AND PHARMACOKINETIC STUDIES OF CANDIDATE HYDROLASES *IN VIVO*.

MAJOR TASK 2: Test candidate hydrolases for toxicity *in vivo.*

☑ **Milestone Achieved: Evaluation of pulmonary toxicity of candidate hydrolase regimens.**

All GH combinations tested were well tolerated by immunocompetent and immunosuppressed mice, with the exception of Ega3. Production of Ega3 was modified and the new protein produced in a mammalian cell line reduced the pulmonary recruitment of eosinophils and other leukocytes following GH therapy, as reported in Major Task 4 (below).

MAJOR TASK 3: Pharmacokinetic studies of candidate hydrolases.

☑ **Milestone Achieved: Evaluation of pharmacokinetics of candidate hydrolase regimens.**

GH half-life varies from 1h (Sph3) to 36h (PslG). Half-lives are generally longer in immunocompromised mice. GH combinations alter the half-live of the proteins in different ways (shorter for PslG, unchanged for PelA, longer for Ega3).

MAJOR TASK 4 (as required): Development of candidate hydrolase variants.

Rationale:
The results of our pharmacokinetics studies suggest that modification of some of the GHs may be required to increase their half-life. The *in vivo* half-life of PslG was estimated above 18h in immunocompetent mice, and 36h in immunosuppressed mice, thus modification of this GH to increase its pharmacokinetic profile is not required at present. In contrast, the 3 other GHs showed half-lives shorter than 12h, especially Sph3 with a half-life of less than 3h in both mouse models. Modification of these GHs may therefore be warranted.

Subtask 2 and 3: Test protease resistance of candidate hydrolases against *A. fumigatus* and *P. aeruginosa* isolates in the epithelial cell damage assay using western blot analysis and mass spectrometry (as required). Cell lines used: A549 epithelial cells [ATCC]. Dr Howell and Sheppard's labs. Months 9-15. Completion level = 100%

Rationale:
Expression of Ega3 in mammalian cell lines has been successfully completed This GH has a higher tolerability in mouse, and comparable enzymatic activity and pharmacokinetics (Subtask 5). We have expressed and purified an orthologue of PelA from *Bacillus cereus.* This protein has a significantly long half-time that PelA (see subtask 5). Modification of Sph3 to improve its half-time has been abandoned (Subtask 4). ~~When~~

Previous accomplishments:
1- Resistance of Sph3 and PelA to the proteases present in biological samples (BAL fluids, mouse lung homogenates and Af293 culture supernatant). As these results did not reproduce our findings of short half-lives of these GHs, we hypothesized that GH therapy could induce the production of pulmonary proteases. However, we found that the injection of Sph3 into healthy mice for 1 day prior to lung harvesting, and subsequent use of the lung homogenates in the protease assay, did not increase the degradation of GHs.

2- Sensitivity of GHs to commercial elastase*:* As previously observed, all GHs were sensitive to elastase. This is of interest since elastase is secreted by mammalian neutrophils and we observed an increase in GH half-life in mice rendered neutrophil deficient. As a consequence of this observation, we choose to assay mammalian neutrophil lysate. Interestingly, the sensitivity was proportional to the measured pharmacokinetics in immunocompetent mice: intact Sph3, PelA and PslG were detectable up to 4h, 6h and more than 24h, respectively, when exposed to elastase, *vs.* half-lives of less than 1h, 3h and 18h in mice. For PslG, the main degradation observed was the removal of the 6-Histine tag as determined by mass spectroscopy.

3- Resistance of GHs to neutrophil lysate*:* Little degradation of PelA or Sph3 was observed before 24h when mixed with neutrophil proteases.

Accomplishments: no accomplishment to report in this period.
While the GH's did not respond as expected to the proteases present in biological fluids such as BAL and lung homogenates, the response to commercial elastase closely matched that of our *in vivo* PK data. The commercial elastase assay will therefore be used as required in the future as the primary screening step for the stability of the GH's.

Subtask 4: Test chemical modification as a means to increase the stability of candidate hydrolases (as required). Dr Howell's lab. Months 9-21. Completion level = 100%

Rationale:
To improve the stability of GHs *in vivo*, we also explored whether chemically attaching polyethylene glycol (PEG) to lysine residues of the GHs would be beneficial. This is one of the most common techniques used to improve stability without affecting the activity of an enzyme.

Previous accomplishments:
PEGylation of Sph3 and PelA gave conflicting results. During the first assay, it did not affect the enzymatic activity of the GHs and provided only limited protection against elastase, and none against trypsin and chymotrypsin. PEGylation only provided significant protection against degradation by papain.

Our second assay focused on elastase due to its importance in neutrophil related proteolytic processes. Surprisingly, PEGylation decreased the half-life of elastase treated Sph3 and PelA. Although this difference could be resolved by repeating the assay, we have chosen instead to assay the PEGylated GHs against leukocyte lysate, which is a more relevant biological assay (see Subtask 3).

The results of the chemical modifications of hydrolases by PEGylation did not show any significant increase in stability of the proteins tested. As other methods (see Subtask 5) of finding more stable variants have proven more successful, we have abandoned the chemical modification approach.

Subtask 5: Test site-specific modification as a means to increase the stability of candidate hydrolases (as required). Dr Howell's lab. Months 9-21. Completion level = 100%.

Previous accomplishments with GHs:

Through genetic engineering, our teams have successfully developed a mammalian HEK293 expression system for Ega3. Ega3 produced in HEK293 cells (Ega3-HEK) was comparable to the *P. pastoris* produced protein in terms of enzymatic activity and pharmacokinetics but significantly more tolerable (significantly less macrophage and eosinophil recruitment in the lungs).

Rationale for developing GH orthologues:

Although this task is formally complete, in an effort to improve GH pharmacokinetics, our labs have been exploring the properties of GH orthologues from other bacterial or fungal species: the Sph3 orthologues from *Aspergillus oryzae* and *Aspergillus nidulans,* and the PelA orthologue from *Bacillus cereus* (BCE5582).

Previous accomplishments with GH orthologues:

Resistance to proteases: Resistance of Sph3 orthologues to elastase was not significantly better than *A. clavatus* Sph3, while BCE5582 demonstrated a higher elastase resistance than

Pseudomonas PelA. We therefore elected to focus our future work on the PelA orthologue BCE5582.

In vitro activity of BCE5582: BCE5582 was produced in Clear coli®. BCE5582 activity against *P. aeruginosa* PA14 biofilm was comparable to *Pseudomonas* PelA. Directed mutation in the putative catalytic site resulted in a dramatic reduction in enzymatic activity, thus confirming that this enzyme shares a similar mechanism of action with PelA.

Pharmacokinetics of BCE5582: An antibody specific for BCE5582 had been generated and used to perform Western blot analyses of mouse lung homogenates following intratracheal BCE5582 administration. The pulmonary half-life of BCE5582 was ~ 8h in immunocompetent mice (experiment performed in duplicate), which is significantly higher than the ~3h half-life of *Pseudomonas* PelA. The results in a single analysis in immunosuppressed mice were much more variable and need to be repeated.

Accomplishments with BCE5582:

Pharmacokinetics: a replicate assay was scheduled for this period, but was delayed due to the unanticipated departure of Ms Melanie Lehoux (research assistant in Sheppard Lab), in September 2019. After advertising the position and interviewing candidates, Ms Mai Nguyen was recruited and joined the Sheppard Lab on January 2020. She will complete the pharmacokinetics assays in coordination with Ms Deepa Raju (research associate in Howell Lab).

Tolerability:
Methodology: a single dose of 500 µg of BCE5582 or PelA was administered intratracheally to immunocompetent mice. Mice were monitored for 7 days, then sacrificed for analysis of pulmonary injury and inflammation. The assay was performed once, with groups of 10 mice per condition: 8 mice had their lungs harvested, lavaged for measurement of lactate dehydrogenase (LDH) release and digested for pulmonary leukocyte recruitment by flow-cytometry. The lungs of the remaining 2 mice were analyzed by histopathology.

Results: No significant alteration in leukocyte recruitment was observed following intratracheal inoculation of BCE5582, except for an increase in pulmonary macrophage numbers that was also seen with PelA (Figure 1). No lung damage was detected by LDH quantification in BAL fluid (Figure 2) or histopathology (data not shown).

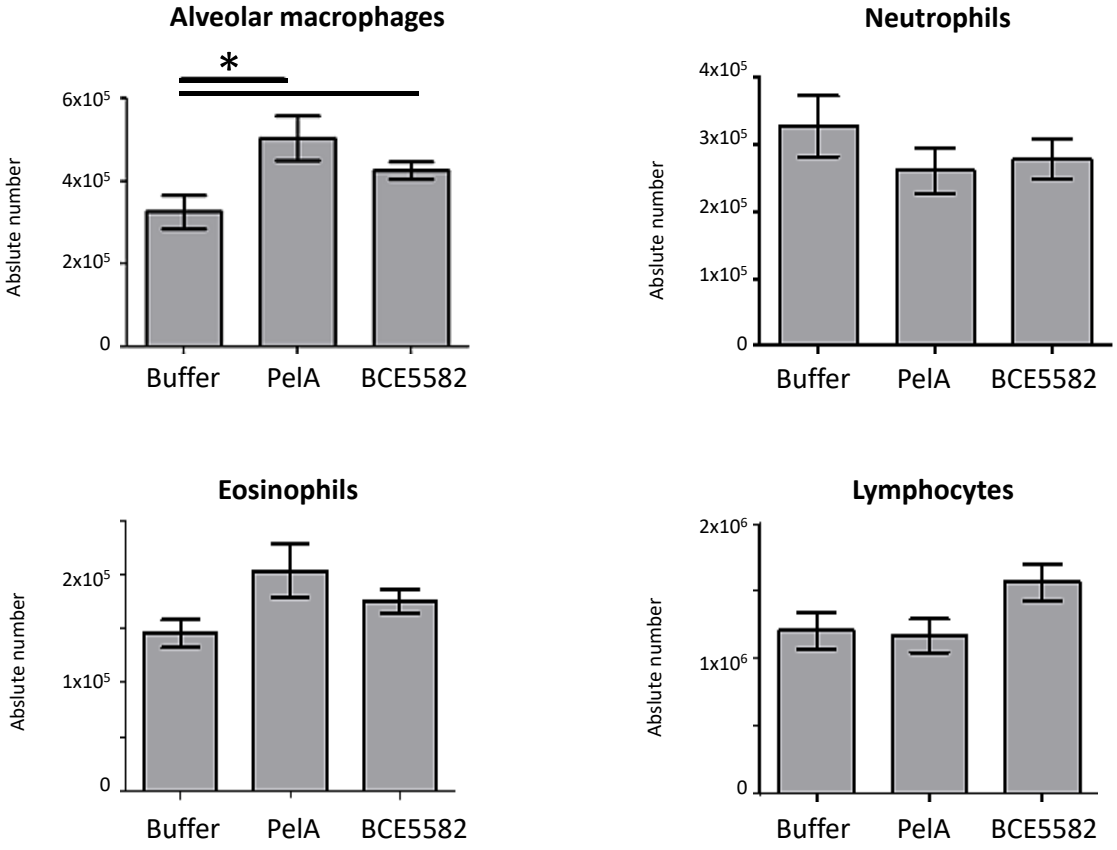

Figure 1. Absolute quantification of leukocyte populations in mouse lungs as measured by flow-cytometry performed on lung digests. Lungs were harvested 7 days following treatment with a single dose of the indicated GH combination. * indicates a significant difference with the untreated group, with $p<0.05$ by ANOVA test.

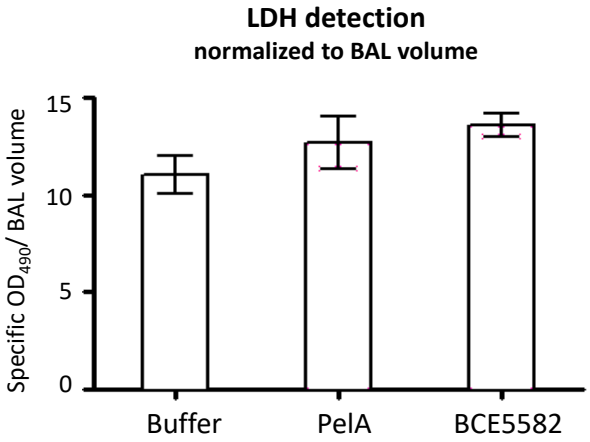

Figure 2. Quantification of lactate dehydrogenase (LDH) activity in bronchoalveolar fluid harvested from mice 7 days following treatment with a single dose of the indicated GH combination. No significant difference was observed between either treatment and the untreated group, with $p\leq0.05$ by ANOVA test.

Efficacy:

Methodology: Groups of 8 mice were rendered neutropenic by treatment with anti-Ly6G antibody (200 µg intraperitoneally every 48 hours beginning 1 day prior to infection). At day 0, a 50 µL suspension containing $5x10^6$ Af293 conidia (wild-type *A. fumigatus*) was administered intratracheally to mice in combination with 500 µg of BCE5582 or sterile GH buffer. Four days after infection, mice were sacrificed and their lungs were harvested for fungal burden estimation by galactomannan (GM) quantification with the commercial kit "Platelia™ Aspergillus EIA" from BioRad. The GM reading was normalized to the weight of the harvested lung and to a highly infected lung homogenate standard (our lab).

Results: A trend towards a reduction of fungal burden in the lungs of infected mice was observed both with PelA and with BCE5582, although it failed to reach statistical significance in this single replicate (Figure 3).

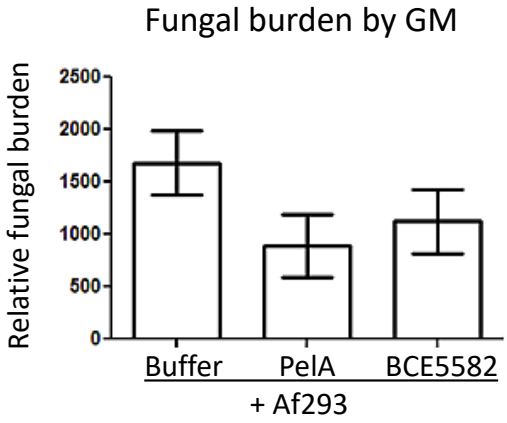

Figure 3. Pulmonary fungal burden of immunosuppressed mice intratracheally infected with $5x10^6$ conidia of *A. fumigatus* and treated with the indicated GH (250 µg single) or the corresponding sterile buffer. No significant difference in GM content as compared with untreated, by ANOVA test.

Although beyond the required SOW, duplicate experiments to test the efficacy of BCE5582 will be performed to confirm the activity of this hydrolase *in viv*o.

❑ **Milestone to achieve: To develop stable candidate hydrolases** (as required)**.**

SPECIFIC AIM 3: TO EVALUATE CANDIDATE HYDROLASES ALONE AND IN COMBINATION WITH ANTIMICROBIAL AGENTS IN THE TREATMENT OF EXPERIMENTAL *A. FUMIGATUS* AND *P. AERUGINOSA* PULMONARY INFECTIONS *IN VIVO*.

MAJOR TASK 5: Test hydrolases for activity in animal models of acute disease. Months 13-30. This major task was completed during previous period.

☑ **Milestone Achieved: Determination of the efficacy of candidate hydrolase regimens in the treatment of acute infection with *A. fumigatus* and *P. aeruginosa*.**

MAJOR TASK 6: Test hydrolases for activity in animal models of chronic disease

Subtask 2: Determine the effects of candidate hydrolase (Ega3) on fungal burden of immunocompetent mice chronically infected with *A. fumigatus*. Dr Sheppard's lab. Months 13-30. This subtask was completed during previous period.

Subtask 3: Determine the effects of candidate hydrolases (PslG/PelA and PslG/Ega3 combinations) on bacterial burden of immunocompetent mice chronically infected with *P. aeruginosa*. Dr Sheppard's lab. Months 18-30. Completion level = 100%.

During the previous grant period, we established the optimal *P. aeruginosa* bead inoculum to establish a non-lethal persistant fungal colonisation that will not be cleared and will not kill the host. Treatment with the combination of 250 µg of PslG and 250 µg PelA in this model did not result in a significant reduction of bacterial burden at day 7, and we elected to focus our efforts on studying the potentiation of antibiotics by GHs in this model chronic *P. aeruginosa* infection (as reported in Major Task 7, Subtask 3)

☑ **Milestone Achieved: To demonstrate efficacy of candidate hydrolase regimens in the treatment of chronic infection with *A. fumigatus* and *P. aeruginosa*.**

MAJOR TASK 7: Test hydrolases for synergy with antimicrobials.

Subtask 2: Determine the effects of hydrolase (Sph3, Ega3, PelA)-antifungal combinations on fungal burden of mice infected with *A. fumigatus*. [10 mice per group X 6 experimental groups X 2 hydrolase antifungal combinations X 2 time points AND 3 mice for histopathology X 6 groups X 2 hydrolase antifungal combinations at a single time point all performed in duplicate = 552 mice] Dr Sheppard's lab. Months 25-36. As per SOW, this task was completed during previous period. In the grant extension period, we proposed to also study the effects of Sph3 and PelA in combination with caspofungin.

Rationale:
Our *in vitro* assays for potentiation of antifungal drugs by GHs (Major Task 1) showed that the most effective GH/antifungal combinations were posaconazole/Sph3 and caspofungin/PelA. Since posaconazole and caspofungin showed comparable potentiation results when assayed *in vitro* with Sph3 as well as with PelA (Major Task 1 Subtask 2), to fulfill the SOW requirements, we

initially elected to assay posaconazole with both GHs. As our previous experiments demonstrated that GH-dependent effects on fungal burden are lost over time, we elected to test two different antifungal/GH combinations using 2 different antifungal concentrations at a single time-point.

1- <u>Potentiation of the effect of posaconazole.</u>

During previous report period:

Using the neutrophil depleted model of invasive aspergillosis, treatment with Sph3 was found to potentiate the antifungal effects of 2.5mg/kg of posaconazole. (Major Task 5 Subtask 3)).

2- <u>Potentiation of the effect of caspofungin.</u>

Methodology: Balb/c mice were neutrophil depleted by intraperitoneal injection of 200 µg of anti-Ly6G antibody every 48h, beginning 1 day prior to infection. At day 0, mice were endotracheally infected with a 50 µL suspension containing $5x10^6$ conidia of Af293 *A. fumigatus*. Mice were then intraperitoneally injected every 24 hours with the indicated dose of caspofungin and monitored daily. Our standard assay of pulmonary GM quantification is not suitable for these studies as caspofungin treatment can result in spurious elevations of GM. Therefore, fungal burden was quantified by quantitative PCR, using the Taqman system and oligonucleotides and probe specific for *A. fumigatus* 18S RNA coding gene.

Results:
During this report period, we performed dose finding experiments to identify a subtherapeutic dose of caspofungin suitable for GH-antifungal combination assays. As has been reported from some groups previously, we were unable to identify a pulmonary fungal burden dose-response curve with caspofungin, even at supratherapeutic doses (Figure 4.)

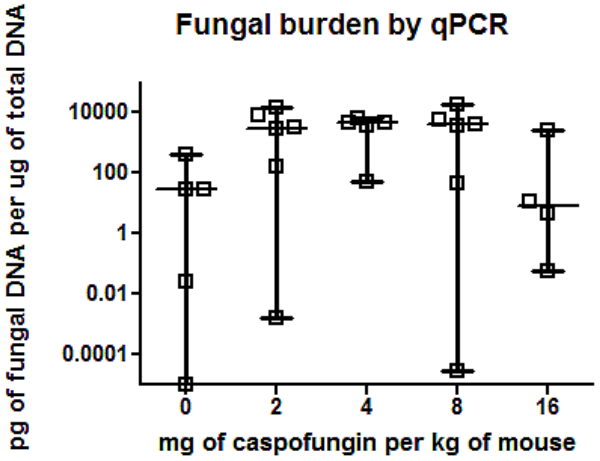

Figure 4. Fungal burden in lungs of immunosuppressed mice intratracheally infected with $5x10^6$ conidia of *A. fumigatus* and treated with the indicated doses of caspofungin. Results shown are from a single experiment with 8 mice per condition. Horizontal lines indicated the median of each experimental group, error bars represent the 75th- and 25th-percentile. No significant change was observed between groups, using the Mann-Whitney test.

We therefore turned to the use of survival as a measure of antifungal effect. A dose of 0.5 and 1 mg/kg of caspofungin were observed to enhance the survival of mice infected with *A. fumigatus*. (Figure 5). We will therefore use a dose of 0.5mg/kg with and without hydrolases in future studies of GH-antifungal efficacy once activities in the lab resume.

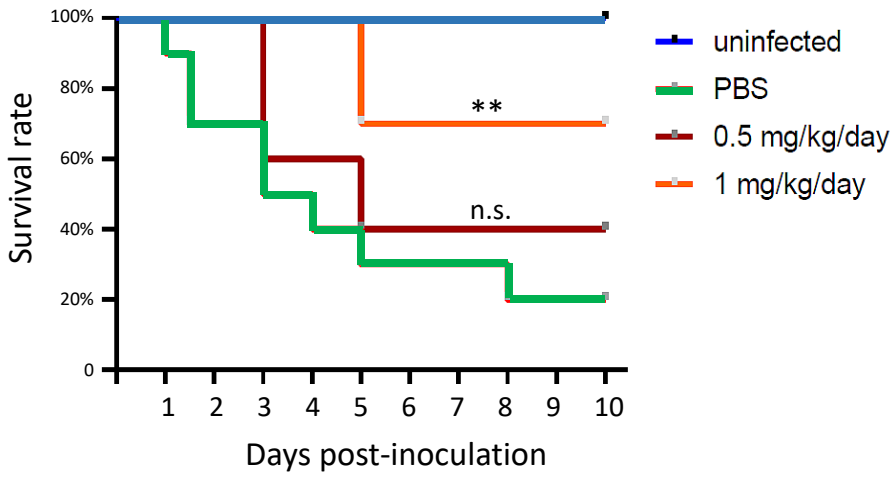

Figure 5. Mouse survival after infection with $5x10^6$ conidia of *A. fumigatus* and daily injection of various doses of caspofungin or with phosphate buffer saline (PBS). ** indicates significant difference for survival with the mice injected with PBS, by Log-rank (Mantel-Cox) test, with $p<0.01$. n.s, indicates no significant difference.

Subtask 3: Determine the effects of hydrolase (PslG/PelA and PslG/Ega3)-antibiotic combinations on bacterial burden of mice infected with *P. aeruginosa*. [10 mice per group X 5 experimental groups X 2 hydrolase –antibiotic combinations X 2 time points AND 3 mice for histopathology X 5 groups X 2 hydrolase –antibiotic combinations at a single time point all performed in duplicate = 460 mice] Dr Sheppard's lab. Months 25-36. Completion level = 100%.

A- Acute model.

1- Potentiation of the effect of ciprofloxacin.

In previous report period, we demonstrated potentiation of the antibacterial activity of ciprofloxacin with the combination of PslG/PelA, but not with either GH alone, or the combination of PslG/Ega3.

2- Potentiation of the effect of ceftazidime:

In previous report period, we identified an optimal ceftazidime dose of 25 mg/kg/day for antibacterial-GH therapy studies.

Accomplishments during this period:

Methodology: Mice were intratracheally infected with a 50 µL suspension containing $1.5x10^7$ bacteria (*P. aeruginosa* PAO1), in combination with 250 µg of each GH in combination, or sterile GH buffer. Ceftazidime was administered intraperitoneally at 4h, 12h and 20h post-infection. At 48 hours post infection, all mice were sacrificed. Blood and lungs were harvested and plated for quantitative culture. These experiments have been performed in duplicate.

Results:

PslG/PelA and PslG/Ega3 combination therapy do not potentiate the effect of ceftazidime.

Treatment with ceftazidime was associated with a reduction in pulmonary bacterial burden (Figure 6A, 7A).

As we have seen previously, treatment with PslG/PelA alone was not associated with a reduction in pulmonary bacterial burden (Figure 6A) and was associated with an increased rate of bacterial hematogenous dissemination (Figure 6B). In contrast to the combination of PslG/PelA with ciprofloxacin, no potentiation of the antibacterial action of ceftazidime by PslG/PelA was observed.

Similar findings were observed with PslG/Ega3 alone and in combination with ceftazidime (Figure 7).

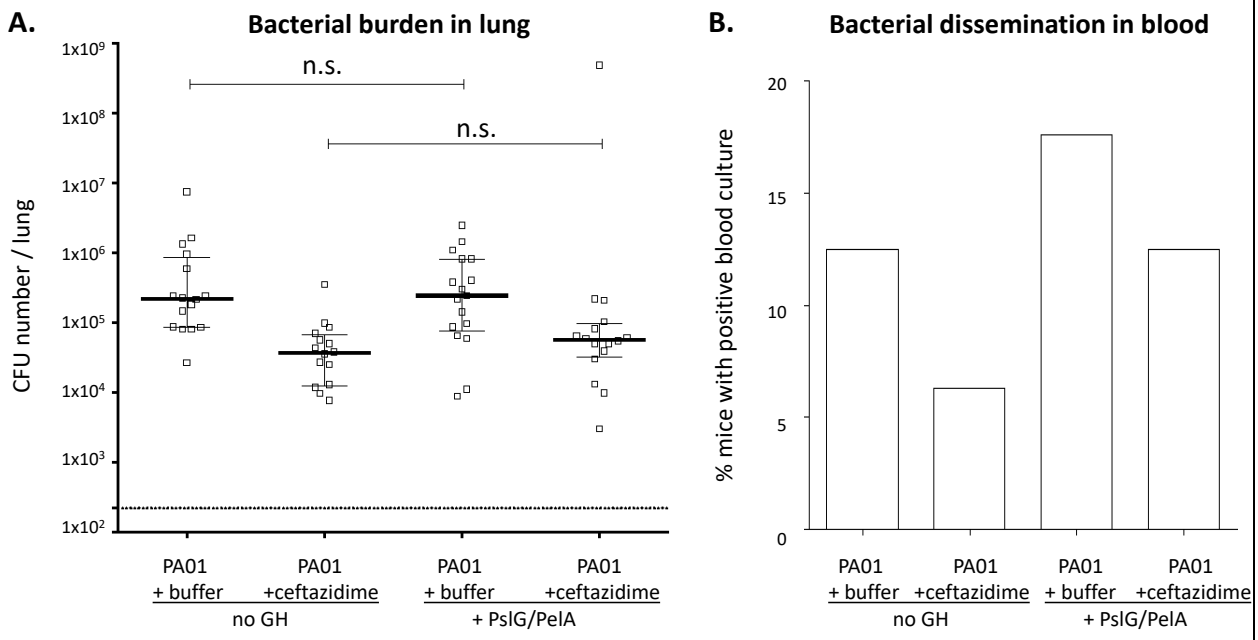

Figure 6. A. Effects of PslG/PelA and ceftazidime on the pulmonary bacterial burden of *P. aeruginosa* infected mice as determined by quantitative culture. B. Percentage of mice with positive blood cultures, in each group. Horizontal lines indicate the median of each experimental group, error bars show the 75th- and 25th-percentile. Experiments were performed on two separate occasions, on groups of 8 mice per condition in each experiment (n=2). n.s. indicate an absence of significant difference in pulmonary bacterial burden between indicated groups, by Kruskall-Wallis test.

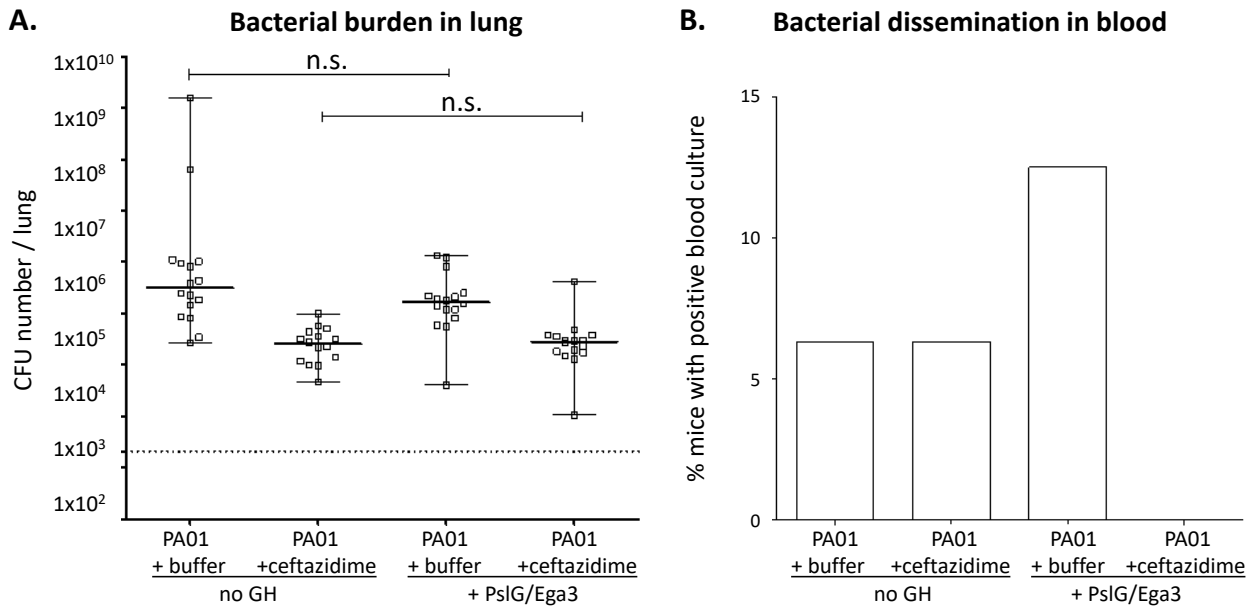

Figure 7. A. Effects of PslG/Ega3 and ceftazidime on the pulmonary bacterial burden of *P. aeruginosa* infected mice as determined by quantitative culture. B. Percentage of mice with positive blood cultures, in each group. Horizontal lines indicate the median of each experimental group, error bars show the 75th- and 25th-percentile. Experiments were performed once, on groups of 8 mice per condition (n=1). n.s. indicates a non-significant difference in pulmonary bacterial burden between indicated groups, by Kruskall-Wallis test.

B- Chronic model.

Although, the SOW required only experiments in the murine acute model of *P. aeruginosa* infection, we extended our experiments to evaluate the effects of GH-antibiotic therapy in the chronic model.

Methodology: Agarose beads were generated by mixing an equal volume of molten agarose solution and bacterial suspension of *P. aeruginosa* strain PA01 (OD_{600nm} = 0.6). Beads of homogeneous size were isolated by passage through 280 µm and 100 µm filters. Beads were quantified by homogenization of an aliquot and quantitative culture on LB medium. Immunocompetent female BALB/c mice were infected intratracheally with a 50 µL agar bead suspension containing 1x10^7 CFUs of *P. aeruginosa*-. At day 2 post-injection, mice were sacrificed and their lungs were harvested and homogenized for quantitative culture was performed. These experiments have been performed in duplicate.

Results:
Neither PslG/PelA nor PslG/Ega3 treatment potentiated the effect of ciprofloxacin (Figure 8A). Haematogenous was much less frequent in the chronic model, with a single mouse developing bacteremia (PslG/PelA treatment alone) in the first replicate (Figure 8B). In light of this, blood cultures were not performed in the second replicate.
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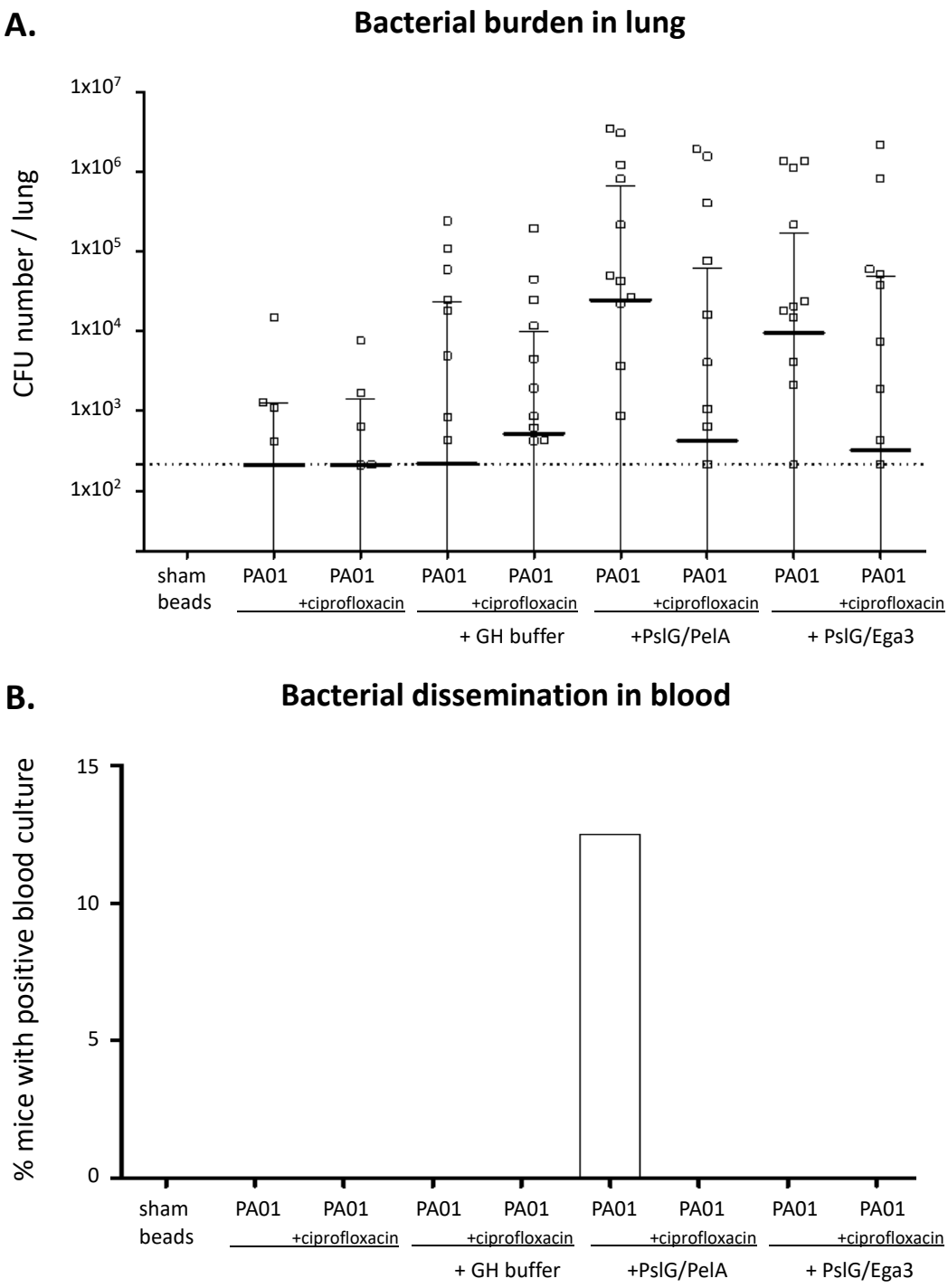


Figure 8. A. Effects of PslG/PelA or PslG/Ega3 and ciprofloxacin on the pulmonary bacterial burden of *P. aeruginosa* infected mice as determined by quantitative culture. B. Percentage of mice with positive blood cultures, in each group. Horizontal lines indicate the median of each experimental group, error bars show the 75th- and 25th-percentile. Experiments were performed on two separate occasions, on groups of 8 mice per condition. No significant difference in pulmonary bacterial burden between groups was identified, by Kruskall-Wallis test.

❑ **Milestone to achieve: To show a proof-of-concept for candidate hydrolases for use in treatment of *A. fumigatus* and *P. aeruginosa*. To make ready to initiate trials of delivery systems and detailed pharmacodynamics experiments as a prelude to Phase I clinical trials.**

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

Mai Nguyen was trained by Rachel Corsini in animal care techniques. She learned to perform intratracheal injection of GHs in mice, as well as mouse infection with pathogens. She also learned to quantify *A. fumigatus* in lung tissues.

How were the results disseminated to communities of interest?

Results were presented at peer conferences, as listed in "6. PRODUCTS. Conferences and presentations."

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

Major Task 4 (as required): Development of candidate hydrolase variants. As outlined above, we have identified a PelA variant, BCE5582, with enhanced protease resistance *in vitro*. We have completed the evaluation of the pulmonary tolerability and tested the efficacy of intra-tracheal BCE5582 therapy in the mouse model of invasive aspergillosis. One replicate of pharmacokinetics studies in the immunosuppressed mice remains to be performed to conclude this work.

Major Task 7: Test hydrolases for synergy with antimicrobials. The GH-antimicrobial combination studies which we have already initiated will be completed with the study of the effects of Sph3 and PelA in combination with caspofungin in the mouse model of invasive aspergillosis.

4. IMPACT

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

Our demonstration that microbial GHs can disrupt biofilms has generated significant interest in the scientific community and other groups have now begun to evaluate microbial enzymes as potential anti-biofilm therapeutics

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

The results of the studies described in this report add value to our existing intellectual property and patent describing the use of microbial GHs as anti-biofilm therapeutics.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS.

Changes in approach and reasons for change

Nothing to report

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

Personal replacement: Progress on Major tasks 4 and 7 have been delayed by the unanticipated departure of Ms Melanie Lehoux (research assistant in Sheppard Lab) in September 2019. After advertising the position and interviewing candidates, Ms Mai Nguyen was recruited and joined the Sheppard Lab on January 2020. She has been trained in fields Ms Lehoux was mastering, and now she is completing the remaining tasks.

All laboratory work in the Sheppard and Howell laboratories was shut down in accordance Provincial guidelines in response to the COVID-19 crisis. A formal research ramp-up date has not yet been confirmed, however we are optimistic that studies will resume in mid-late June.

Almost all Major Tasks are now complete. There has not been deviation from the originally proposed studies, as described in the originally submitted Statement of Work, subsequent progress reports and extension requests. All animal use protocols for this work are approved and up to date. We foresee completing the remaining portion of the study within the additional time extension, if we are allowed to restart our research activities.

Changes that had a significant impact on expenditures

Nothing to Report

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

Significant changes in use or care of human subjects

No use of human subjects in this grant

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.

Journal publications

Nothing to Report

Books or other non-periodical, one-time publications.

No publication in books or other non-periodical to be reported

Other publications, conference papers, and presentations.

Oral presentation at conferences:

P.L. Howell:
Invited speaker, Sarkar Symposium, Research Institute, The Hospital for Sick Children, Toronto, Canada. Feb 20 2020. *Microbial biofilms: Mechanisms to potential therapeutics*.

D.C. Sheppard:
Invited Speaker, Advances against Aspergillosis and Mucormycosis, Lugano, Switzerland. March 3 2020. Aspergillus biofilms*: informing new therapies*.

Invited Speaker, Cidara Expert Insight Series, San Diego, California. Jan 21 2020. *Novel Antifungal therapies*.

Invited Speaker, CIFAR Program: Fungal Kingdom, Threats and Opportunities, Toronto, Canada. Nov 17, 2019. *Novel therapies for fungal infections through a glycobiology lens*.

Invited Speaker, ID week 2019, Washington DC. Oct 6 2019.*New science behind invasive aspergillosis*.

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

No dissemination of the results through a website to be reported

- **Technologies or techniques**

No new technology to be reported.

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

Patent

Howell PL, Baker P, Alnabelseya N, Sheppard DC, Bamford N, Little D, Snarr B, United States Provisional Patent application (No. 62/008,836) entitled "Soluble Bacterial and Fungal Proteins and Methods and Uses Thereof in Inhibiting and Dispersing Biofilm". National phase filing in US, Canada, Europe, Australia and Japan occurred between Dec 2016 – Jan 2017 (Actual date depends on jurisdiction). Patent prosecution is currently ongoing in all jurisdictions.

- **Other Products**

No other product to be reported

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

For "person month worked" purpose, please note that this Annual report covers a 8 month period, from October 15th, 2019, to May 14th, 2020, rather than a 12 month period.

Na*me:* *P. Lynne Howell*
Project Role: PI
Research Identifier 0000-0002-2776-062X
Nearest person month worked 2
Contribution to Project: Responsible for the research performed at The Hospital for Sick Children.

Name: *Deepa Raju*
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 7
Contribution to Project: Dr Raju is co-responsible for protein expression and purification, as well as responsible for antibiotic potentiation and pharmacokinetic assays.

Name: *Piyanka Sivarajah*

Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	7
Contribution to Project:	Ms Sivarajah is responsible for protein expression and purification.

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

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

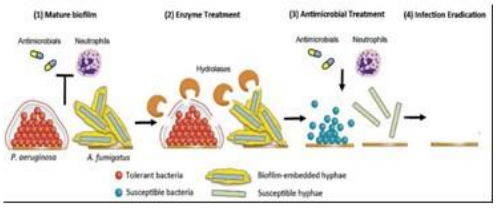
8. SPECIAL REPORTING REQUIREMENTS

AWARD CHART.

Award# W81XWH-16-1-0284 Log# PR150786-P1: **Development of New Therapeutics Targeting Biofilm Formation by the Opportunistic Pulmonary Pathogens *Pseudomonas aeruginosa* and *Aspergillus Fumigatus*.**

PI: Pr Lynne Howell, Hospital for Sick Children, Toronto (ON), Canada
Budget: $972,320.00 Topic Area: Respiratory Health
Mechanism: Peer Reviewed Medical Research Program, Investigator-Initiated Research Award, Partnering PI Option, W81XWH-15-PRMRP-IIRA

Research Area(s): **Award Status:** 15-SEP-2016 to 14-MAY-2020



Study Goals:

A. fumigatus and *P. aeruginosa* are two lung opportunistic pathogens that embed themselves in a biofilm, becoming therefore more resistant to drugs and host defenses. We will test the use of four therapeutic enzymes, two glycosyl hydrolases (GH) from fungal origin, two from bacterial origin, to render microorganisms more susceptible to antimicrobials *in vivo*. We will determine the concentration of hydrolases that are both efficient and well tolerated by the host. Our purpose is to conduct proof of concept studies to move these agents into early clinical trials.

Specific Aims:

Aim 1. To characterize the ability of microbial GHs to enhance the activity of antimicrobial agents *in vitro*.
Aim 2. Perform preliminary tolerability and pharmacokinetic studies of candidate hydrolases *in vivo*.
Aim 3. To evaluate candidate hydrolases alone and in combination with antimicrobial agents in the treatment of experimental *A. fumigatus* and *P. aeruginosa* pulmonary infections in vivo. Demonstrate proof-of-concept for candidate hydrolases for use in treatment of *A. fumigatus* and *P. aeruginosa*.

Key Accomplishments and Outcomes:

☑ Routine production of recombinant GHs in sufficient quantity and quality for all experiments
☑ GH + antimicrobial combinations with synergistic activity against *A. fumigatus* and *P. aeruginosa* *in vitro* have been identified.
☑ Doses of up to 500 µg of Sph3, PelA and PslG produced in *E. coli* and Ega3 produced in HEK293 cells are well tolerated intratracheally by mice
☑ Pulmonary GH half-lives vary from 1-18h (immunocompetent mice), and 3-36h (immunosuppressed mice). GH degradation may be leukocyte-dependent.
☐ The development and evaluation of protease-resistant GH variants allowed the identification of a *Bacillus cereus* PelA orthologue (BCE5582) that showed similar enzymatic activity *in vitro* and similar efficacy *in vivo,* but higher tolerability and longer half-life in lungs (studies are being completed).
☑ Sph3, PelA, and Ega3 monotherapy reduces *A. fumigatus* pulmonary fungal burden in lungs and improves survival while PelA, PslG, and Ega3 alone and in combination do not reduce *P. aeruginosa* pulmonary burden and enhance hematogenous dissemination.
☑ In chronic infection models: no GH, in monotherapy or in GH combinations, has been able to decrease microbial burden in lungs, even when combined with ciprofloxacin in the treatment of chronic *P. aeruginosa* infection.
☐ in invasive aspergillosis, Sph3 and PelA failed to potentiate the antifungal activity of posaconazole; study of caspofungin potentiation by GH is ongoing. In *P. aeruginosa* infection, PslG/PelA but not PslG/Ega3 potentiated the effect of ciprofloxacin, while neither GH combination potentiated the effect of ceftazidime.
☐ Ready to initiate trials of delivery systems and detailed pharmacodynamics experiments as a prelude to Phase I clinical trials.

Publications: **2017**: Snarr BD *et al.*, **PNAS**, 10.1073/pnas.1702798114; Zhang S *et al.*, **Cell Microbiol**. 10.1111/cmi.12799. **2018**: Snarr BD *et al.*, **Future Microbiology**, 0.2217/fmb-2017-0243; Little DJ *et al.*, **Plos Pathogens**, 10.1371/1006998; Asker D *et al.*, **Biomaterials**, 10.1016/j.biomaterials.2018.03.016; Low K *et al.*, **Curr Opin in Struct Biol**, 10.1016/j.sbi.2018.05.001. **2019**: Speth C *et al.*, **Virulence** 10.1080/21505594.2019.1568174; Ostapska H *et al.*, **PLOS Pathogens**, 10.1371/1007411; Bamford NC et al, **J Biol Chem – Editor's pick**, 10.1074/jbc.RA119.009910; Zacharias CA *et al.*, **Curr Opin Microbiol**, 10.1016/j.mib.2019.04.006; Le Mauff F *et al.*, **J Biol Chem**, 10.1074/jbc.RA119.008511.

Patents: Howell PL *et al.*, United States Provisional Patent application (No. 62/008,836) entitled "Soluble Bacterial and Fungal Proteins and Methods and Uses Thereof in Inhibiting and Dispersing Biofilm".

Funding Obtained: none to date

9. APPENDICES.