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

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14. ABSTRACT

Major accomplishments this period:
1. Pharmacokinetic studies of the GH enzymes were completed (Major Task 3). GH variants (Major Task 4) were developed for Sph3, Ega3 and PelA, to address their short half-life *in vivo* (PelA, Sph3). A test for GH resistance to neutrophil lysate and to the commercial protease elastase was developed. , Native and variant GHs show sensitivity to neutrophil lysate and elastase. PEGylation protects the proteins from elastase. The PelA orthologue showed identical activity and increased resistance to elastase and improved pharmacokinetics. The Sph3 orthologue showed no improvement.
2. Survival of immunosuppressed mice challenged with *A. fumigatus* was not observed in a chronic model of aspergillosis (Major Task 6) suggesting that GHs are inefficient against an established fungal infection. Conversely, intra-tracheal injection of any of the GHs was protective in an acute model of aspergillosis (Major Task 5). But combination of GHs (Sph3 and PelA) with posaconazole failed to improve the outcome of acute disease further than the effect of the antifungal or of the GH alone (Major Task 7). Study with caspofungin is upcoming.
3. Conversely to *Aspergillus* infection, GH alone failed to improve the outcome in mice challenged with *P. aeruginosa* in an acute model (Major Task 5); au contraire, the results of these studies indicate that GH therapy alone may increase bacterial dissemination, in particular towards blood, thus worsening the infection. The addition of PslG, PelA, Ega3 or PslG/Ega3 to ciprofloxacin failed to potentiate the antibiotic activity, although these combination of GH/antibiotic protected against bacterial dissemination. One exception is the addition of PslG/PelA combination to ciprofloxacin: it potentiated the decrease of bacterial burden by ciprofloxacin (Major Task 7); this suggests the need of a GH against Pel plus one GH against Psl. Data from the chronic model are being produced (Major Task 6).

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

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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

<u>MAJOR GOALS FOR YEAR 3:</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 II.***

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 II.***

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

Subtask 1: Express and purify Sph3, Ega3, PelA and PslG for subtasks 2 – 4. (Months 6-12) (PI: Howell) ***Achieved Year I.***

Subtask 2: Test pharmacokinetics of hydrolases (Sph3, Ega3, PelA and PslG/PelA and PslG / Ega3 combinations) in immunocompetent mice. (Months 6-12) (PI: Sheppard)

Subtask 3: Test pharmacokinetics of hydrolases (Sph3, Ega3, PelA and PslG/PelA and PslG/ Ega3 combinations) in immunosuppressed mice. (Months 6-12) (PI: Sheppard)

Subtask 4: Determine concentrations of candidate hydrolases and their combinations using animal tissue samples. (Months 6-12) (PI: Howell)

Milestone: Evaluation of pharmacokinetics of candidate hydrolase regimens. (Month 12)

<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 I.***

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 II.***

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

Subtask 1: Express and purify Sph3, Ega3, PelA and PslG for subtasks 2 – 5 (Months 13-30). (PI: Howell) ***Achieved Year I.***

Subtask 2: Determine the effects of hydrolases (Sph3, Ega3, PelA) on survival of immunosuppressed mice infected with *A. fumigatus.* (Months 13-30) (PI: Sheppard)

Subtask 3: Determine the effects of hydrolases (Sph3, Ega3, PelA) on fungal burden of mice infected with *A. fumigatus*. (Months 13-30) (PI: Sheppard) ***Achieved Year II.***

Subtask 4: Determine the effects of hydrolases (PslG/PelA and PslG/Ega3 combinations) on bacterial burden of mice infected with three strains of *P. aeruginosa.* (Months 13-30) (PI: Sheppard).

Milestone: 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 I.***

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)

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 I.***

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)

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 3:

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*.**

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.**

MAJOR TASK 3: Pharmacokinetic studies of candidate hydrolases.

Previous accomplishments in this Major Task:

Polyclonal antibodies that recognize Sph3, PelA or PslG were produced by our group prior to the start of this project. The necessity to change Ega3 production from *E. coli* to *P. pastoris*, then to the mammalian cell line HEK293, required the preparation of a new anti-Ega3 antibody for the completion of following Subtasks 2, 3 and 4. A polyclonal anti-Ega3 antibody was successfully produced and characterized during the first year of this grant; it recognizes Ega3 produced by both *P. pastoris* and HEK293. Antibodies were used for Western-blot detection of recombinant GHs in mouse pulmonary homogenates following intratracheal injection of each GH .

At the time of previous Annual Technical Report, the GHs had shown a wide range of pulmonary half-lives, from less than 1h (Sph3) to more than 48h (PslG) when administered as monotherapy.The PslG/Ega3 combination remained to be tested as we shifted production of Ega3 from *P. pastoris* to the HEK293 human cell line. PslG/PelA had been assayed only once.

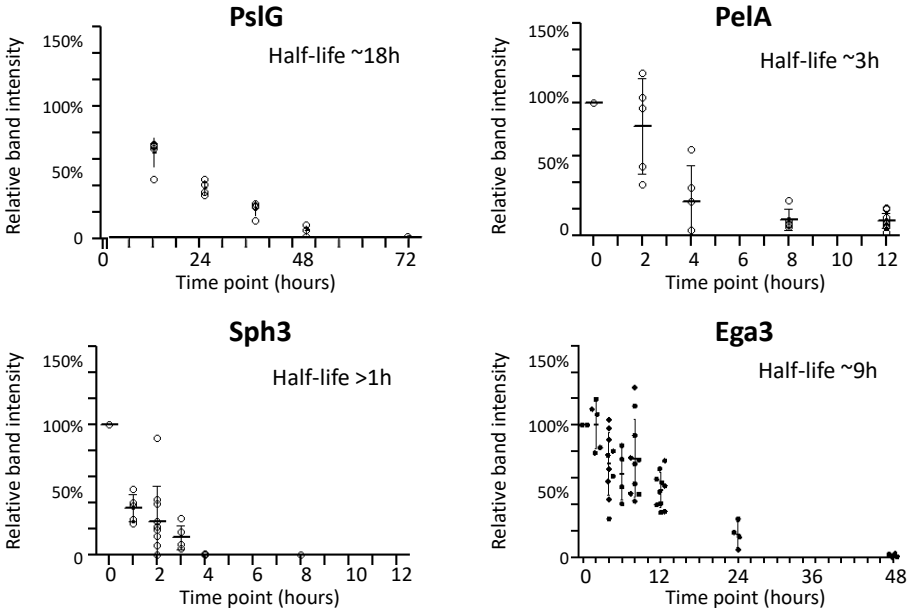
Subtask 2: Test pharmacokinetics of GHs (Sph3, Ega3, PelA and PslG/PelA and PslG/Ega3 combinations) **in immunocompetent mice** [25 mice per group (5 per time point) X 5 GH therapies; 1 group of 25 untreated mice. All performed in duplicate = 300 mice]. Dr Howell and Sheppard's labs. Combined with **Subtask 4**: **Determine concentrations of candidate hydrolases and their combinations using animal tissue samples**. Dr Howell's lab. SOW Time Period: Months 6-12. Completion level = 100%

Accomplishments:

Methodology: GHs were administered intratracheally to BALB/C female mice at a dose of 500 µg for single GHs or 250 µg of each GH if they were injected in combination. At time points ranging from 1 to 48 h after GH administration, the mice were sacrificed and their lungs harvested and homogenized in phosphate buffer and a cocktail of protease inhibitors. Lung homogenates were analyzed by Western-blotting using rabbit anti-GH antibodies. A goat-anti-rabbit-HRP antibody was used as the secondary antibody; HRP signal was detected and analyzed by densitometry whereby the percent band intensity was normalized to the total band intensity at the 0h time point for each mouse. A minimum of 4 mice were used for each time point in each experiment. Following the results of the first experiment, earlier or later time-points were added as required to encompass the half-life of each GH.

Results: The results presented in Figure 1 are the combination of all mice studied in this task. A third replicate of the PslG/PelA combination was performed as an unusually long half-life of PelA was noted when combined with PslG when it was first tested (as reported in October 2018); this finding was not reproduced however in the 2 following replicates, and was considered an outlier.
Interestingly, the half-life of some GHs (PslG, PelA and Sph3, but not Ega3) was extended in leukopenic mice, suggesting that degradation of GHs may at least in part be leukocyte-dependent.

Immunocompetent mice



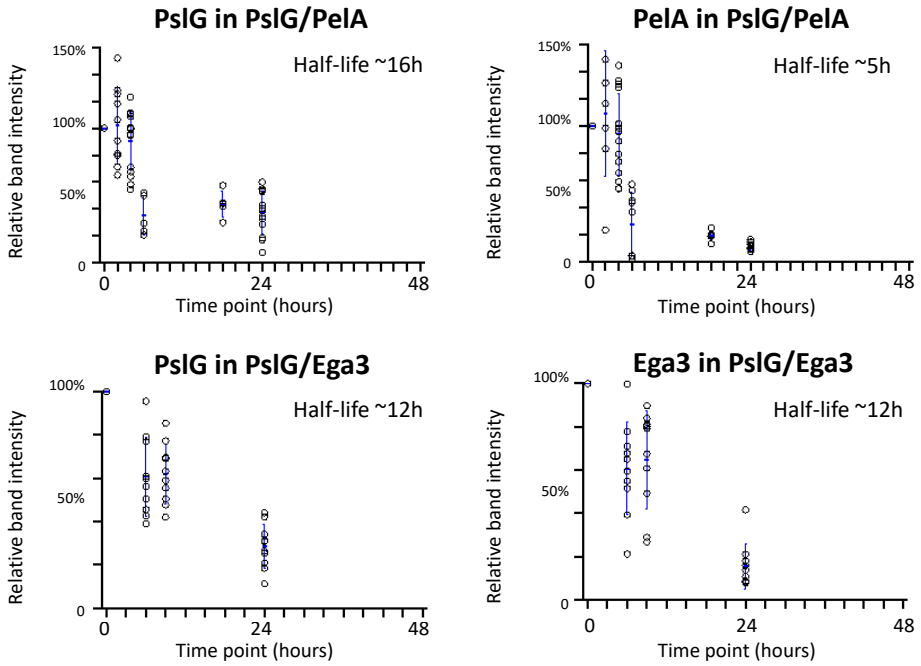


Figure 1. Determination of pulmonary GH pharmacokinetics by Western-blot analysis. GHs were intratracheally administered to immunocompetent mice at a dose of 500 µg of GH if injected alone, or as a mix of 250 µg of each GH if injected as a GH combination. Each square represents the densitometry value from a single mouse. Results are corrected for background signal obtained from untreated mouse lung samples and normalized to the total band intensity at 0h for each GH. Graphs represent the compilation of all experiments performed during the grant period.

Subtask 3: **Test pharmacokinetics of hydrolases** (Sph3, Ega3, PelA and PslG/PelA and PslG/Ega3 combinations) **in immunosuppressed mice** [25 mice per group (5 per time point) X 5 hydrolase therapies; 1 group of 25 untreated mice. All performed in duplicate = 300 mice. Howell and Sheppard's labs. Combined with **Subtask 4**: **Determine concentrations of candidate hydrolases and their combinations using animal tissue samples**. Dr Howell's lab. SOW Time Period: Months 6-12. Completion level = 100%.

Accomplishments:

Methodology: Two days prior to treatment, mice were immunosuppressed by injection of 250 mg/kg cortisone subcutaneously and 250 mg/kg cyclophosphamide intraperitoneally. GH injection, lung treatment and pharmacokinetics were performed as described above (Subtask 2).

Results: The results of these pharmacokinetic studies indicated that, with the exception of Ega3, all GH proteins exhibited a longer, half-life in immunosuppressed mice as compared with immunocompetent mice (Figure 2).

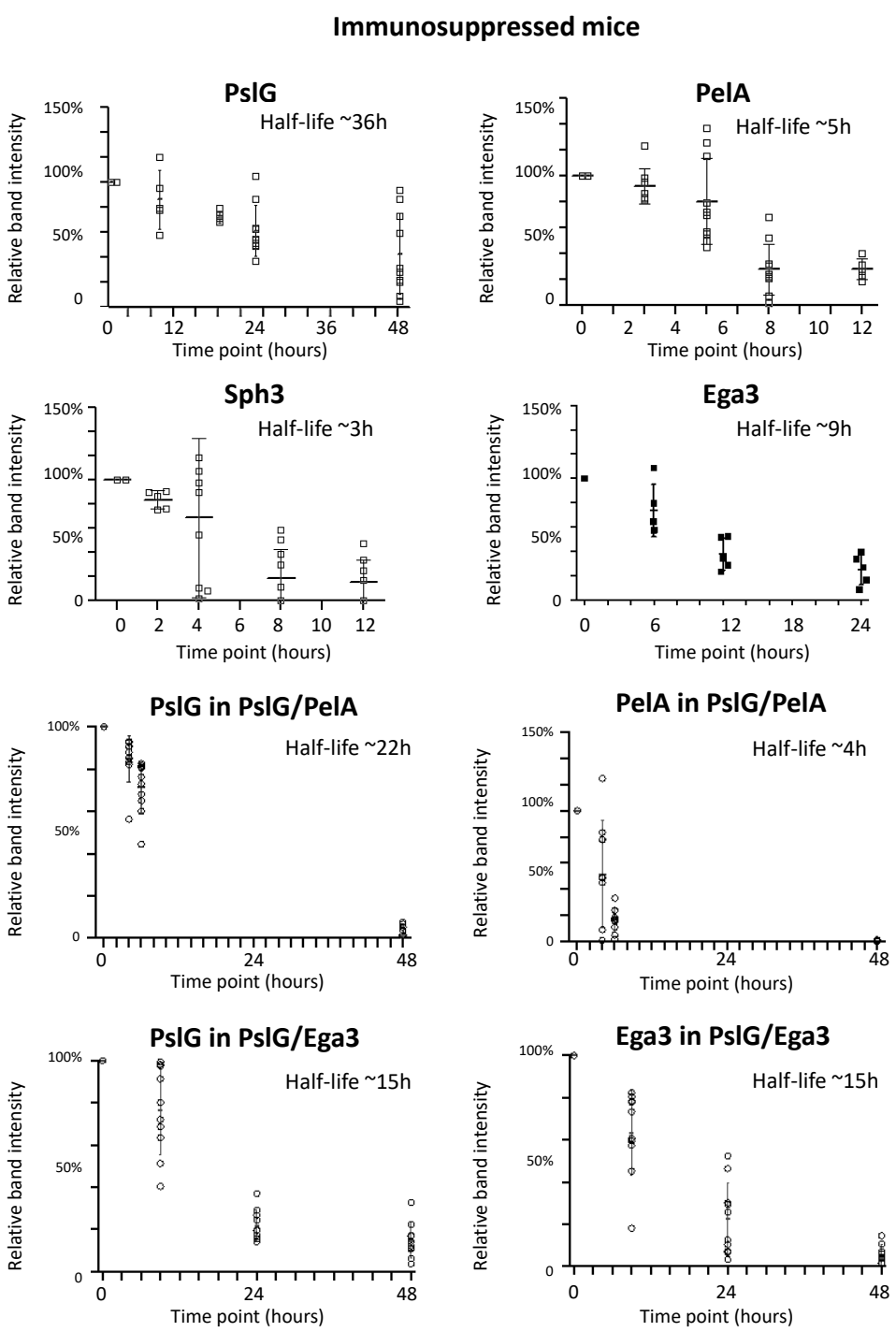


Figure 2. Determination of pulmonary GH pharmacokinetics by Western-blot analysis GHs were intratracheally administered to immunosuppressed mice at a dose of 500 µg of GH if injected alone, or as a mix of 250 µg of each GH if injected as a GH combination. Each square represents the densitometry value from a single mouse. Results are corrected for background signal obtained from untreated mouse lung samples and was normalized to the total band intensity at the 0h time point for each GH. Graphs represent the compilation of all experiments performed during the grant period.

Table 1 summarizes the final pharmacokinetics of GHs as determined by all experiments in **Subtasks 2, 3 and 4**.

Hydrolase	PslG	PelA	Ega3	Sph3	PslG/PelA		PslG/Ega3	
					PslG	PelA	PslG	Ega3
Estimated half-life in immunocompetent mice (Subtask 2)	18h	3h	9h	1h	16h	5h	12h	12h
Estimated half-life in immunocompromised mice (Subtask 3)	36h	5h	9h	3h	22h	4h	15h	15h

Table 1. Estimation of half-life of GHs in mouse lung following intratracheal injection of 500 μg of pure GH in PBS or a combination of 250 μg of each of two GHs administered simultaneously.

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

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 (Table 1). 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 = 30%

Rationale:

Genetic modifications of Ega3 have been successfully completed, generating a GH with a higher tolerability in mouse and comparable enzymatic activity and pharmacokinetics (Subtask 5), but modifications of Sph3 and PelA, to improve their half-time, are still ongoing (Subtask 4). When Subtask 4 is complete, we will test the protease resistance of all resulting GH variants.
In preparation for these experiments we have been developing an *in vitro* protease resistance assay to test the susceptibility of the GH's to proteases from diverse sources: commercial (proteinase K, elastase) or biological (BAL fluids and lung homogenates lung homogenate from mice, fungal culture supernatants, neutrophil lysate). We also have been testing our *in vitro* protease resistance assay against an orthologue of PelA produced by *Bacillus cereus.*

Previous accomplishments in the subtask:

In previous experiments, we showed that Sph3 and PelA were resistant to the proteases present in BAL fluids, in 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.

Accomplishments:

1- Resistance of GHs to commercial elastase.

Methodology: 1 µg of GH was incubated at 37 °C with 60 µl of phosphate buffer saline (PBS) with or without elastase. At the indicated time points, GH degradation was stopped by adding SDS-PAGE buffer and samples were analyzed by SDS-PAGE and Western-blotting.

Results: As previously observed, all GHs were sensitive to elastase (Figure 3). 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 (Table 1). For PslG, the main degradation observed was the removal of the 6-Histine tag as determined by mass spectroscopy.

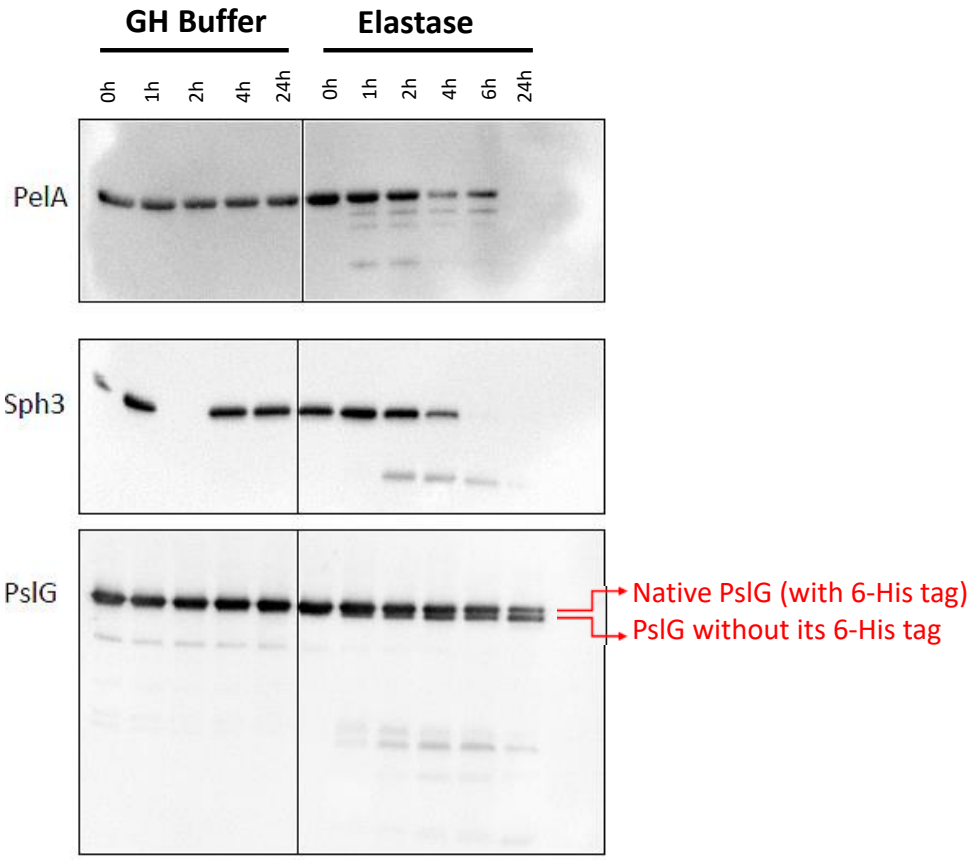


Figure 3: Western-blot monitoring of PelA, Sph3 and PslG persistence when incubated with elastase.

These data confirmed that GHs are sensitive to commercial 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.

2- Resistance of GHs to neutrophil lysate.

Methodology: Cells from the HL60 cell line (derived from peripheral blood leukocytes in a human patient with acute promyelocytic leukemia) were propagated *in vitro*, then differentiated into neutrophils by exposure over 3 days to dimethylsulfoxide (DMSO, 1.3%) and retinoic acid (2.5 µM). Differentiated cells were then activated by 1 h exposure to N-formylmethionyl-leucyl-phenylalanine (fMLP, 100 nM), concentrated to $2x10^7$ cells/ml by gentle centrifugation and stored at -80°C until required. On the day of the protease assay, a frozen sample was thawed, vortexed, and sonicated or treated with RIPA buffer (150mM NaCl, 5mM EDTA, 50mM Tris pH 8, 1% NP40 and 0.1% SDS) to facilitate lysis of the cells. Cell debris were removed by centrifugation and the supernatant used in the proteolytic assay as cell lysate. A mix of 1 µg of protein and 100 µL of HL60 lysate or control buffer was incubated at 37°C. At the indicated time points, GH degradation was stopped by adding SDS-PAGE buffer and samples were analyzed by SDS-PAGE and Western-blotting with our specific anti-GH antibodies.

Results: Little degradation of PelA or Sph3 was observed before 24h (Figure 4). It is possible that the neutrophil proteases released from $2x10^7$ cells are not sufficient to degrade the GHs. In order to optimize this assay, in future experiments we will use lysates from a higher number of cells.

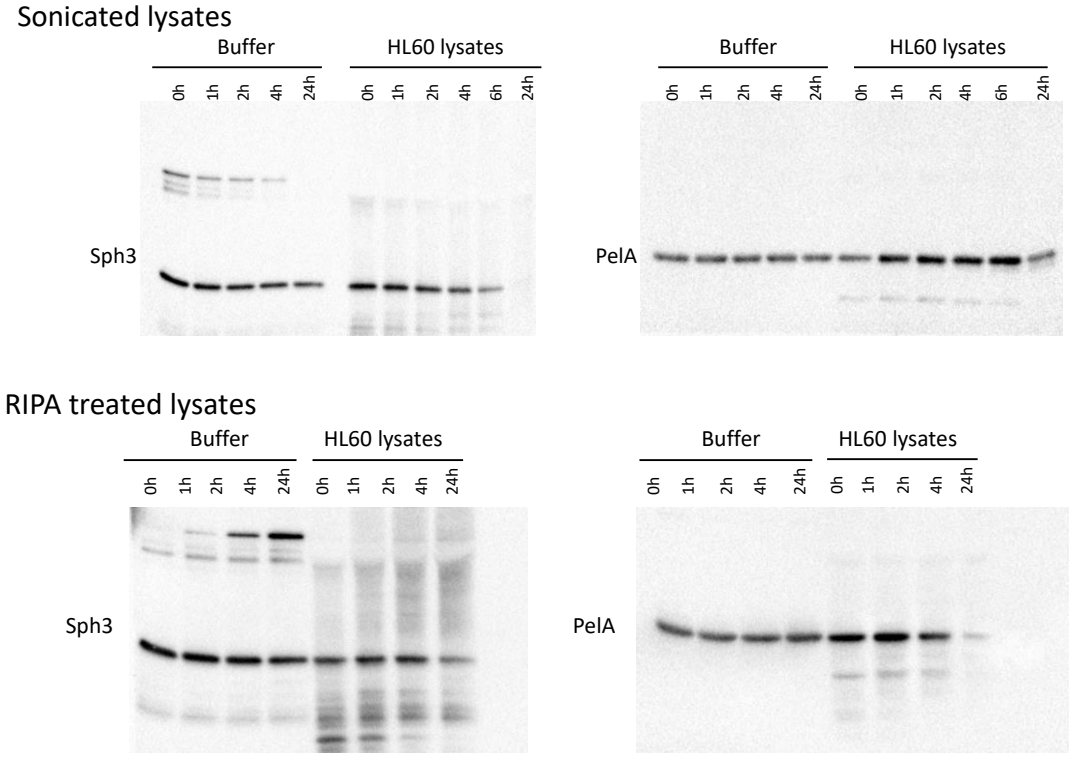


Figure 4: Western-blot monitoring of *in vitro* PelA and Sph3 persistence when incubated with neutrophil lysates (HL60 lysates).

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 = 20%

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 was confirmed through a molecular weight shift on SDS-PAGE. PEGylated GHs remained enzymatically active as seen in biofilm disruption assays. During a first assay, PEGylation conferred a 1h protection to both Sph3 and PelA when assayed against elastase, while it protected the GHs against degradation by papain but not against trypsin and chymotrypsin.

Accomplishments:

Methodology: PEGylation of GHs was performed by using a commercial kit (EZ-Link™ NHS-PEG4 Biotinylation Kit) following manufacturer's instructions. GH modification was visualized on SDS-PAGE as a shift in the migration of the enzyme. Lungs of uninfected mice were collected, resuspended in PBS without protease inhibitors and homogenized. Elastase was resuspended in PBS at 100 mg/mL. For the protease resistance assay, 1 µg of GH was incubated at 37 °C with 60 µl of either lung homogenate or PBS with or without elastase at 1/1 000. At the indicated time points, GH degradation was stopped by adding SDS-PAGE buffer and samples were analyzed by SDS-PAGE and Western-blotting.

Results: Given previous results only elastase was assayed due to its importance in neutrophils related proteolytic processes, and because it was the only protease that didn't degrade the GHs rapidly. In the second assay in contrast to our previous results, we found that PEGylation decreased the half-life of elastase treated Sph3 and PelA by approximately 1 hour (Figure 5). Although we could resolve this difference by repeating the assay for a third time, we have chosen to instead to assay the PEGylated GHs against leukocyte lysate, which is a more relevant biological assay (see Subtask 3).

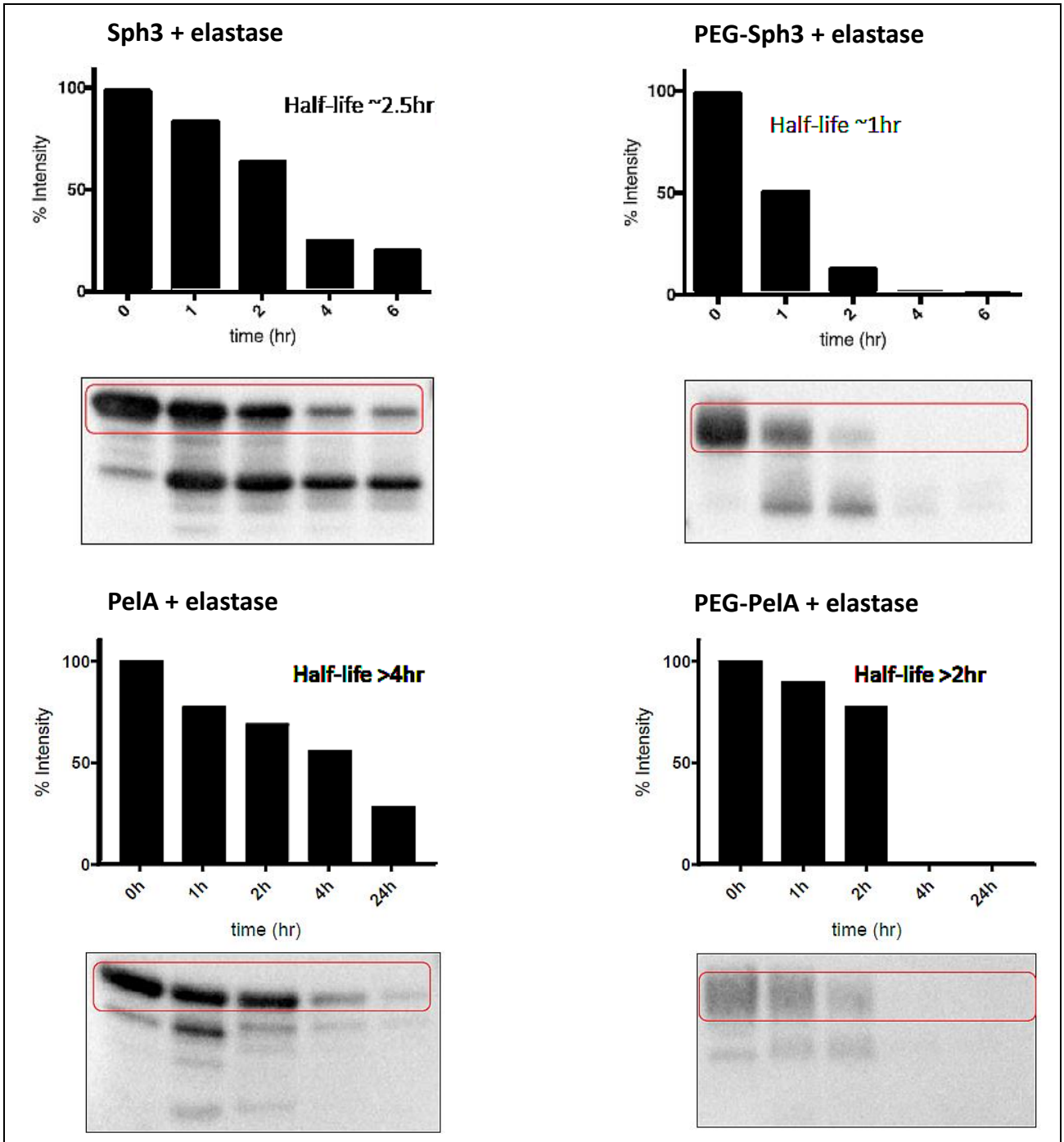


Figure 5. Western-blot monitoring and band density study of Sph3, PelA and their PEGylated versions persistence when incubated with elastase.

Previously, the proteases contained in the lung homogenate failed to degrade Sph3 and PelA. The PEGylated version of these GHs showed the same resistance (Figure 6).

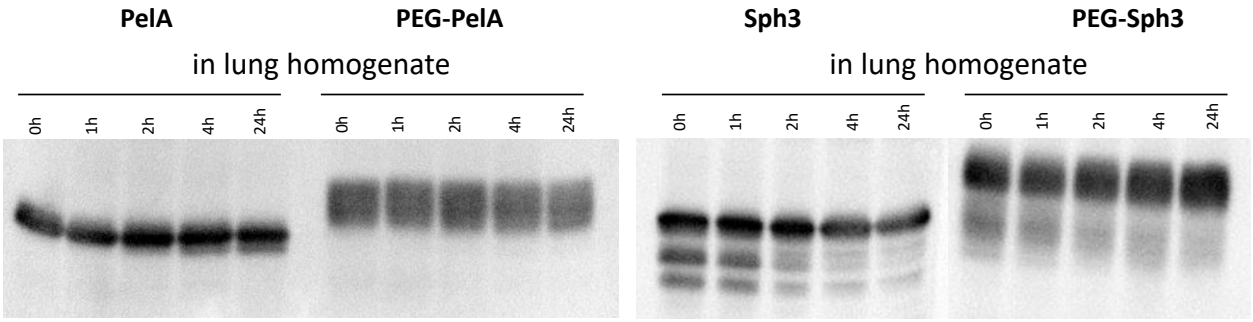


Figure 6. Western-blot monitoring of the persistence of Sph3, PelA and their PEGylated versions, in lung homogenates obtained from non-infected mice.

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:

Through genetic engineering, our teams have successfully developed a mammalian HEK293 expression system for Ega3. As a result, HEK293 produced Ega3 is comparable to the *P. pastoris* produced protein in terms of enzymatic activity and pharmacokinetics, while it has mammalian-like N-glycans and was found to be significantly more tolerable (significantly less macrophage and eosinophil recruitment in the lungs).

Rationale:

Although this task is completed under SOW requirements, in an effort to improve GH pharmacokinetics, our labs have been exploring the properties of GH orthologues from other bacterial or fungal species.

Accomplishments:

A. PelA orthologue from *Bacillus cereus*.

1- Production of BCE5582.

The gene *BCE5582* had been identified in a strain of *Bacillus cereus* as coding for an orthologue of PelA and was PCR amplified and cloned into a pET24 vector with a His tag on the C-terminal. The plasmid was transformed into Clear coli® for expression. For protein purification, cultures were grown in TB, induced with IPTG overnight and centrifuged. The bacterial pellet was sonicated, and the protein purified using nickel affinity chromatography similar to other hydrolases.

2- Activity of BCE5582.

Methodology: BCE5582 activity was measured using our standard biofilm disruption assay: a mature *P. aeruginosa* PA14 biofilm was exposed to either PelA, BCE5582 or to the corresponding buffer. After 1h incubation and washes with PBS, the remaining biofilm was stained by crystal violet, then destained. The biofilm biomass was expressed as OD_{595nm} of the destaining solution.

Results: *In vitro* assays showed that BCE5582 efficiently degrades established *P. aeruginosa* PA14 biofilm (Figure 7), with an EC_{50} that is comparable to *Pseudomonas* PelA.

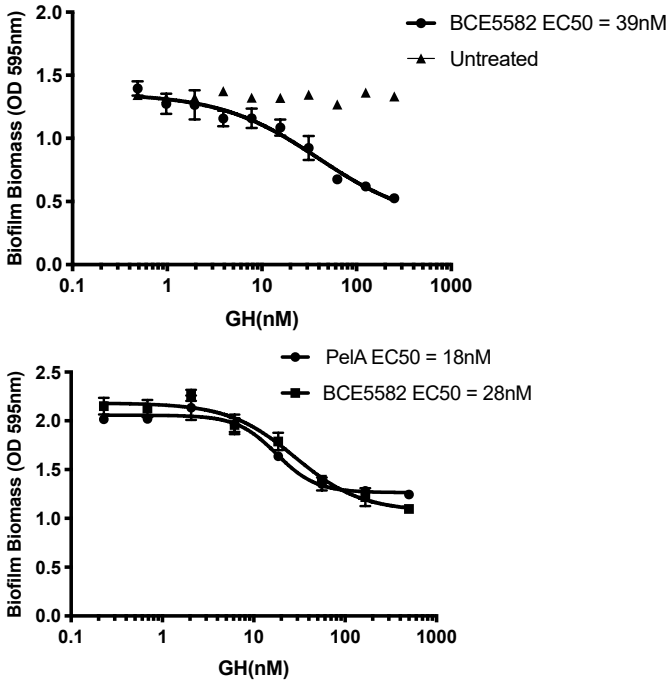


Figure 7: Compared enzymatic activity of PelA and BCE5582, as measured by *P. aeruginosa* PA14 biofilm disruption assay.

3- Alteration of BCE5582 active site.

Methodology: To generate the E213A point variant of BCE5582, PCR mutagenesis was performed to generate a BCE5582 variant which encoded a replacement of a glutamate by an alanine at position 213 of the enzyme.

Results: BCE5582 degraded *P. aeruginosa* biofilm as efficiently as PelA (EC_{50} are comparable) (Figure 8). Therefore, we had started to study its properties through mutation of various amino-acids to demonstrate that the enzyme activity of BCE5582 is specifically responsible for the disruption of biofilm. We mutated the predicted catalytic residue E218, an active site residue. Replacement of this glutamate with alanine resulted – as expected – in a dramatic drop in enzymatic activity.

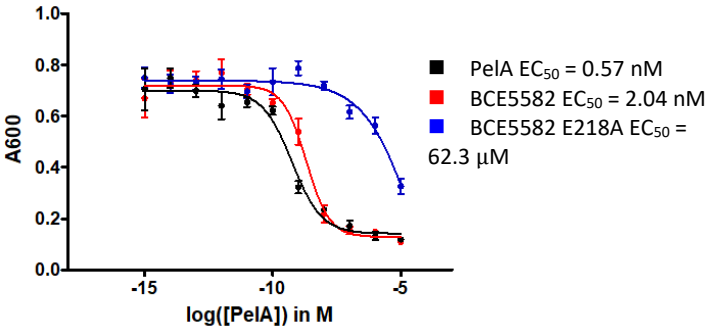


Figure 8: Enzymatic activity of PelA, BCE5582, and BCE5582 mutant ($BC5582^{E218A}$) as measured by *P. aeruginosa* PA14 biofilm disruption assay.

4- Resistance of BCE5582 to commercial elastase.

Protease assay: 1 µg of GH was incubated at 37 °C with 60 µl of phosphate buffer saline (PBS) with or without elastase. At the indicated time points, GH degradation was stopped by adding SDS-PAGE buffer and samples were analyzed by SDS-PAGE.

Results: the resistance of BCE5582 to elastase was significantly higher than for *Pseudomonas* PelA. BCE5582 showed no significant degradation after 6h of incubation with elastase, while degradation of PelA was observed after only 1h (Figure 9). Given that BCE5582 degraded biofilms a similar EC_{50} to PelA, and exhibited increased resistance to elastase we proceeded to examine its pharmacokinetics *in vivo.*

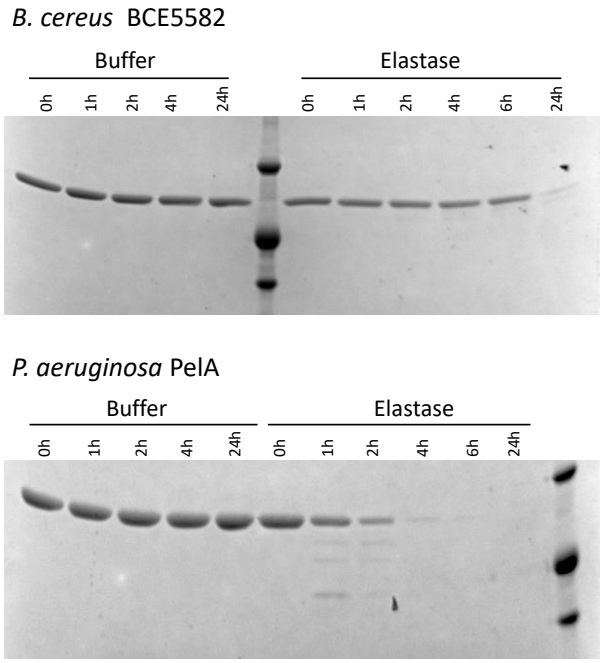


Figure 9: Western-blot monitoring of *in vitro* PelA and BCE5582 persistence when incubated with elastase.

5- Pharmacokinetics of BCE5582.

Given the increased resistance of BCE5582 to elastase in vitro, we proceeded to test pulmonary the pharmacokinetics of this protein, using antibody specific for BCE5582 that has just been generated by our teams.

Methodology: Determination of GH pulmonary pharmacokinetics in immunocompetent and immunocompromised mice was performed as described above (Major Task 3 Subtask 2).

Results: The pulmonary half-life of BCE5582 was ~ 8h in immunocompetent mice, significantly higher than the ~3h half-life of PelA (Figure 10). The pulmonary half-life of BCE5582 in immunosuppressed mice was not calculable due to variability of the results, and a repeat experiment will be required.

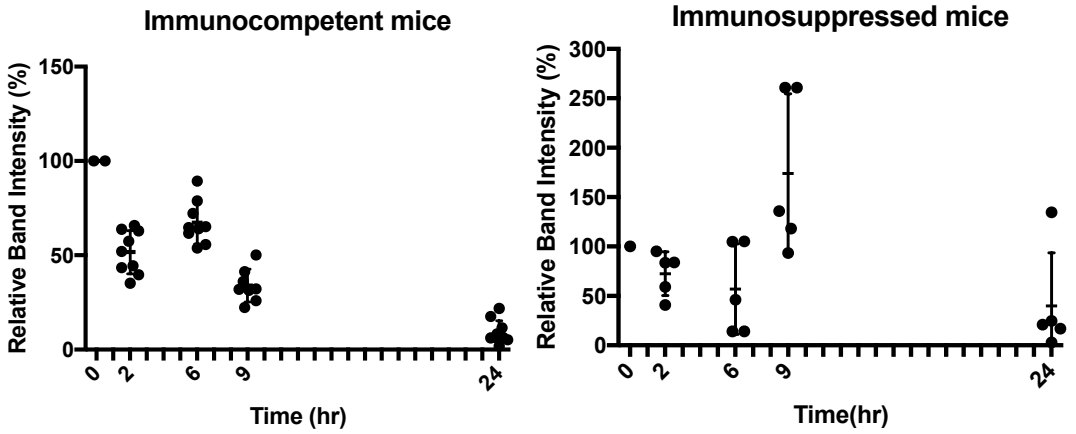


Figure 10: Determination of pulmonary GH pharmacokinetics by Western-blot analysis. 500µg of BCE5582 was intratracheally administered to immunocompetent or immunosuppressed mice. Each symbol represents the densitometry value from a single mouse. Results are corrected for background signal obtained from untreated mouse lung samples and was normalized to the total band intensity at the 0h time point for each GH. Graphs represent n=2 for immunocompetent mice and n=1 for immunosuppressed mice

B. Sph3 orthologue from Aspergillus spp.

Sph3 demonstrated the shortest half-life (~1h in immunocompetent mice). Given the lack of solubility of recombinantly expressed *A. fumigatus* Sph3, we have been using *A. clavatus* Sph3 for our studies. To find a more stable protein we have screened Sph3 orthologues from other *Aspergillus* spp and evaluated them for stability and efficacy.

1- Production of Sph3 orthologues.

Sph3 orthologue genes identified from 2 different species of Aspergillus, *A. oryzae* and *A. nidulans* were cloned into a pET28 expression vector for expression and purification of a His-tagged protein in *E. coli* similar to Sph3 from *A. clavatus* that was used for the in vivo studies. Proteins were purified using a Nickel His -trap gravity column as previously described.

2- Resistance of Sph3 orthologues to commercial elastase.

Elastase stability assays showed that the Sph3 orthologues from *A. oryzae* and *A. nidulans* had comparable stability to the *A. clavatus* Sph3 enzyme (Figure 11). All three Sph3's were susceptible to elastase degradation within 2h of exposure to the protease. In future studies, beyond the scope of this SOW, we will look at identifying residues to mutate to generate more stable variants of the protein.

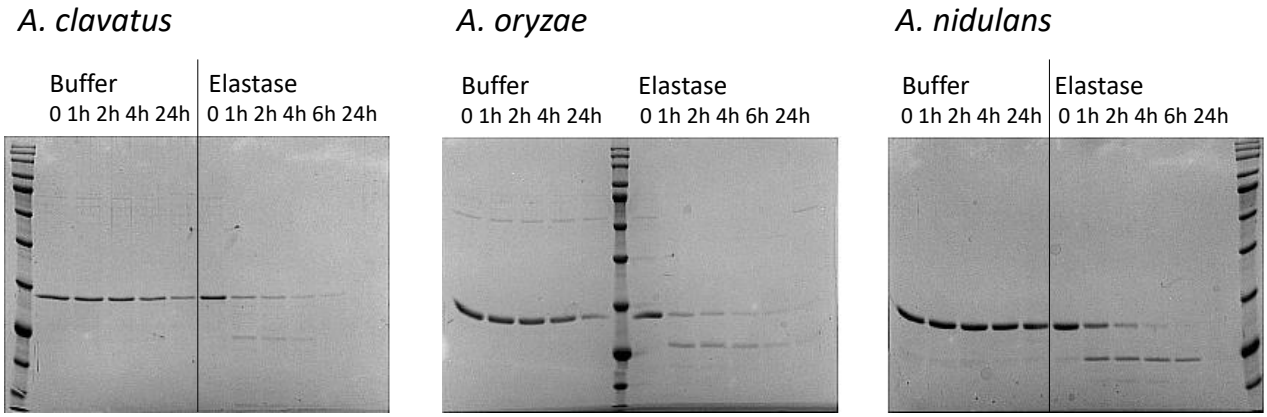


Figure 11: Western-blot monitoring of *in vitro* PelA and Sph3 orthologue persistence when incubated with elastase

Given that Sph3 orthologues resistance to elastase is no significantly better than the one of *A. clavatus* Sph3, we will not proceed further with these orthologues at present.

❑ **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

Previous accomplishments:
The effect of intratracheal treatment with a single dose of GHs (Sph3, Ega3, PelA) on the fungal burden of mice infected with *A. fumigatus* was studied in Subtask 3. In these studies, we found that a single dose of 500 µg of any of the GHs, as well as treatment with GH combinations (250 µg of each GH), resulted in a significant reduction in pulmonary fungal burden 4 days after *A. fumigatus* challenge. GH therapy reduced fungal burden to levels observed in mice infected with a GAG-deficient strain of *A. fumigatus* (Δ*uge3*), suggesting that these enzymes efficiently degrade GAG *in vivo* to attenuate virulence. **(Subtask 3: completed**).

Subtask 2: Determine the effects of hydrolases (Sph3, Ega3, PelA) **on survival of immunosuppressed mice** infected with *A. fumigatus*. Dr Sheppard's lab. SOW Time Period: Months 13-30. Completion level = 100% [10 mice per group X 8 experimental groups X 3 hydrolase regimens, AND 3 mice for histopathology all performed in duplicate = 624 mice]

Previous accomplishments:

Previous studies demonstrated that in a model of acute invasive aspergillosis, Sph3 treatment had no significant effect on mouse weight, temperature, leukocyte recruitment or survival. These results were unexpected since we observed that Sph3 was able to induce a significant reduction in pulmonary fungal burden at 4 days post-infection (Major Task 5 Subtask 3). Given the short half-

life of Sph3 (Major Task 3), we hypothesized that the antifungal effects of a single dose therapy with Sph3 were likely lost after 4 days. Therefore, a less acute model of invasive aspergillosis or a multi-dose treatment may be required to study the effects of GH therapy on survival.

Previously, in our 2018 annual report, the effects of GH therapy on the survival of mice in a neutrophil-depleted mouse model of invasive aspergillosis (using the anti-Ly6G antibody) instead of our more aggressively immunosuppressed mouse model (using cortisone + cyclophosphamide) were reported. In a first experiment, we reported that Sph3 treatment resulted in a trend towards increased survival of infected mice, but this failed to reach statistical significance. During the last year, we replicated the experiment with Sph3 and expanded it to two other GHs, PelA and Ega3.

Accomplishments:

Methodology: Mice were rendered neutropenic by treatment with anti-Ly6G antibody (200 µg intraperitoneally every 48 hours, beginning 1 day prior to infection). Mice were then infected intratracheally with a 50 µL suspension of $5x10^6$ Af293 or Δ*uge3* conidia, or sterile conidial buffer, with or without 500 µg of the appropriate GH. Mice were monitored daily and euthanized upon reaching clinical endpoints. Experiments were performed on two separate occasions, with groups of 8 mice per condition.

Results: As was observed in the first experiment with Sph3, co-administration of PelA, Ega3 or Sph3 at the time of *A. fumigatus* infection significantly increased the survival of neutropenic mice (Figure 12). These results, combined with the reduction in fungal burden previously demonstrated during GH therapy, provide solid pre-clinical evidence for the use of GH therapy in invasive aspergillosis.

Of note, as the overall mortality of Af293 infected GH untreated mice did not reach 100% in these experiments, a repeat experiment utilizing a dose of 10^7 conidia of strain Af293 was performed. However this increased inoculum resulted in complete mortality of all mice by day 2 (data not shown - reported to DOD as an unexpected result). Therefore, all further experiments were performed with an inoculum of $5x10^6$ conidia of strain Af293.

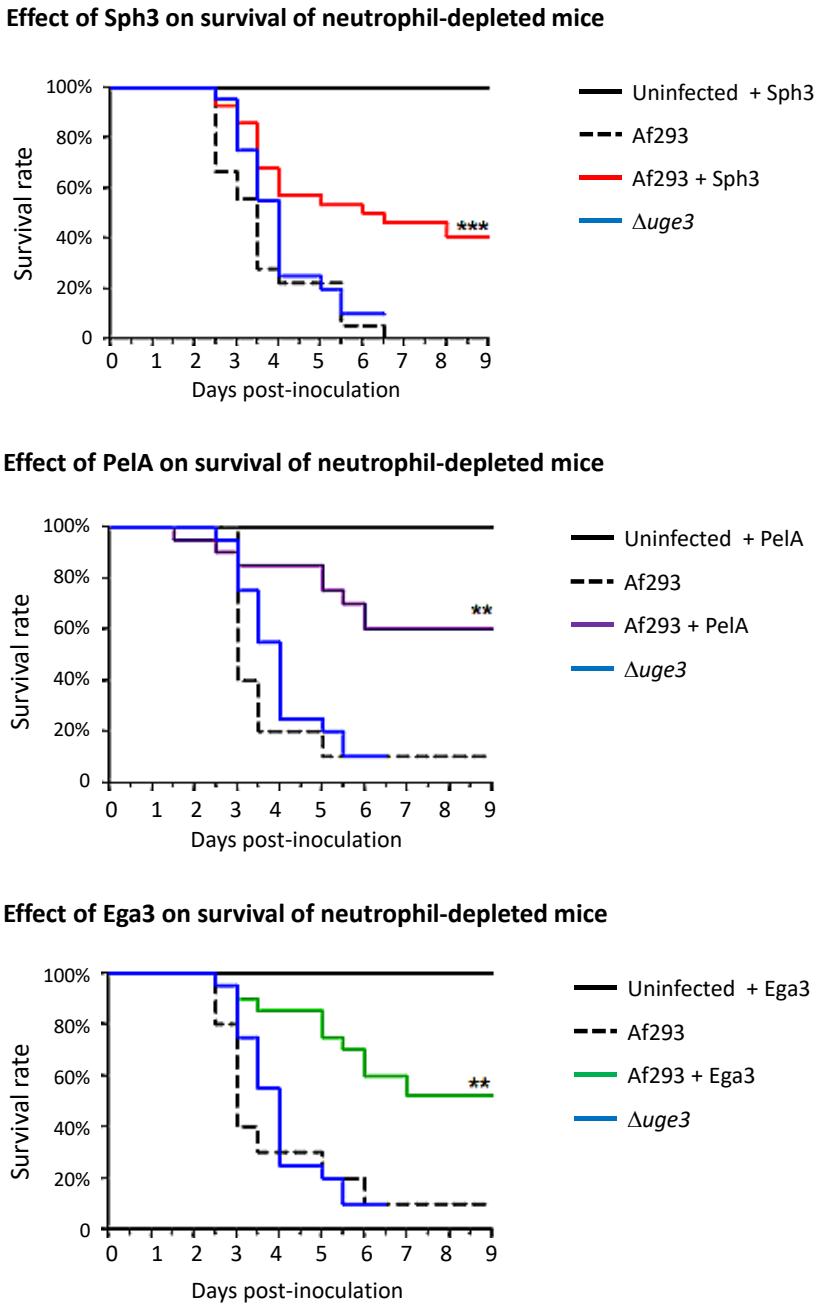


Figure 12. Survival of neutropenic mice inoculated intratracheally with a single dose of $5x10^6$ conidia of the indicated *A. fumigatus* strain or the corresponding sterile buffer in the presence or absence of 500 µg of the indicated GH. Experiments were performed in triplicate for Sph3 and in duplicate for PelA and Ega3, with 8 mice per group in each experiment. ** and *** indicate a significant change in survival with respectively $p<0.01$ and <0.001 by the Wilcoxon-rank test as compared to mice infected with Af293 alone

This subtask is now complete.

Subtask 3: Determine the effects of hydrolases (Sph3, Ega3, PelA) on fungal burden of mice infected with *A. fumigatus*. This subtask was completed during year 1.

Subtask 4: Determine the effects of hydrolases (PslG/PelA and PslG/Ega3 combinations) on bacterial burden of mice infected with three strains of *P. aeruginosa*. Dr Sheppard's lab. Months 13-30. Completion level = 100%. [10 mice per group X 7 experimental groups X 2 hydrolase regimens X 3 strains X 2 time points AND 3 mice for histopathology X 7 groups X 2 hydrolase regimens X 3 strains at a single time point all performed in duplicate = 1932 mice]

Previous accomplishments.

Studies were initiated with PslG monotherapy in Year 1. Unexpectedly, during two separate experiments, mice infected with *P. aeruginosa* PA01 in combination with intratracheal PslG exhibited a trend towards higher pulmonary bacterial compared to the mice that received PAO1 alone. PslG treatment was also associated with dissemination of the bacteria in blood (not observed with PA01 alone), and with increased eosinophil but decreased neutrophil and lymphocyte recruitment to infected lungs. GH combination therapy was deferred in favor of the following mechanistic studies. As expected, we observed that PslG increased the spreading of motile bacteria (twitching motility assay) in a dose-dependent manner, but had no effect on non-motile strains. In contrast, bacterial burden in lungs, bacterial dissemination in blood, and leukocyte recruitment to the lungs were similar when an inactive PslG variant (where we had modified the catalytic site) was used instead of native PslG, as well as when *P. aeruginosa* PA01 was replaced by a bacterial strain deficient in Psl production (i.e. lacked the PslG substrate).
Taken together, these results indicate that although Psl plays a role in lung colonization by *P. aeruginosa*, PslG alone is not protective against *P. aeruginosa* infection. Possible explanations are that PslG may upregulate other bacterial virulence factors in response to PslG exposure or be active on host glycans, leading to tissue injury and increased dissemination.

Accomplishments:

1- Effects of PelA therapy on the virulence of *P. aeruginosa in vivo*.

Methodology: Mice were treated intratracheally with a 50 µL suspension containing $1.5x10^7$ bacteria (*P. aeruginosa* PAO1), combined with 500 µg PelA or the sterile GH buffer, as indicated. At 48 hours post-infection, all mice were sacrificed; blood and lungs were harvested and plated for quantitative culture.
PelA monotherapy resulted in increased pulmonary bacterial burden and hematogenous dissemination (Figure 13). These observations were similar to those previously reported with PslG therapy, suggesting that these effects may be generalizable to multiple GHs.

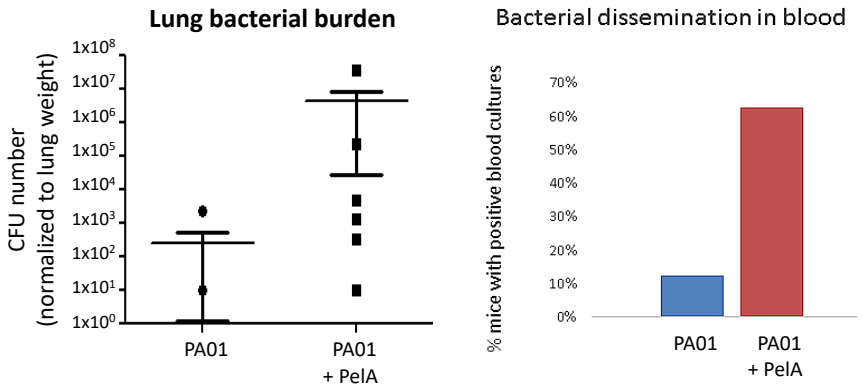


Figure 13. Effect of PelA on pulmonary bacterial burden and hematogenous dissemination of bacteria as determined by quantitative culture of lung homogenate and blood, respectively. Results shown are from a single experiment.

In light of these findings, a series of supplemental experiments were performed to probe the mechanisms underlying the effects of GH therapy on *P. aeruginosa* dissemination.

2- Effects of PslG treatment on twitching motility of Psl deficient *P. aeruginosa* strains *in vitro*.

Methodology: 1% LB-agar with or without PslG (0.05 or 2 µM as final concentration) were stab inoculated with approximately $4x10^8$ of each bacterial strain. After 24 hours of incubation at 37°C, petri dishes were stained with crystal violet and washed. The degree of bacterial spread was measured as an indication of twitching motility.

Results: Consistent with our *in vivo* findings, PslG treatment enhanced motility of both Psl-producing and Psl-deficient strains in a dose-dependent manner (Figure 14). Moreover, treatment of both strains with Sph3, which has no activity against *P. aeruginosa* Psl, also enhanced the motility of *P. aeruginosa*, suggesting that the effect of GH enzymes on motility is independent of their catalytic activity. This finding was consistent with our previously reported observation that both active and catalytically inactive PslG induced similar levels of increased pulmonary bacterial burden and haematogenous dissemination.

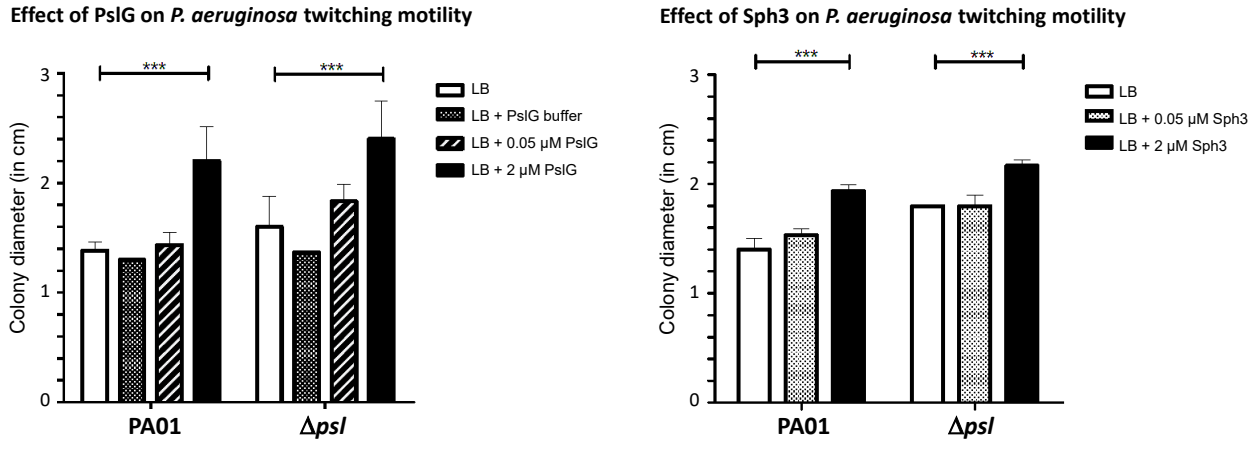


Figure 14. Effect of PslG and Sph3 on the motility of *P. aeruginosa.* Bacterial strains were point-inoculated on a nutrient agar plate with or without PslG at 0.05 or 2 µM. Bacterial spreading was measured as proportional to the diameter of the biofilm attached to the plastic of the plate. *** indicates a significant difference with the untreated group with $p<0.05$ by ANOVA.

2- Effects of protein supplementation on *P. aeruginosa* twitching motility *in vitro*.

Methodology: 1% LB-agar alone, or supplemented with PslG (2 µM as final concentration, equivalent to 0.1 mg/mL) or bovine serum albumin (BSA, at 0.1 mg/mL) was stab inoculated with approximately $4x10^8$ of each bacterial strain. After 24 hours of incubation at 37°C, petri dishes were stained with crystal violet and washed. The degree of bacterial spread was measured as an indication of twitching motility.

Results: As previously seen, PslG enhanced the motility of wild-type and Psl-deficient *P. aeruginosa*. BSA supplementation also enhanced motility of both *P. aeruginosa* strains, although the effect was less marked than was seen with PslG (Figure 15).

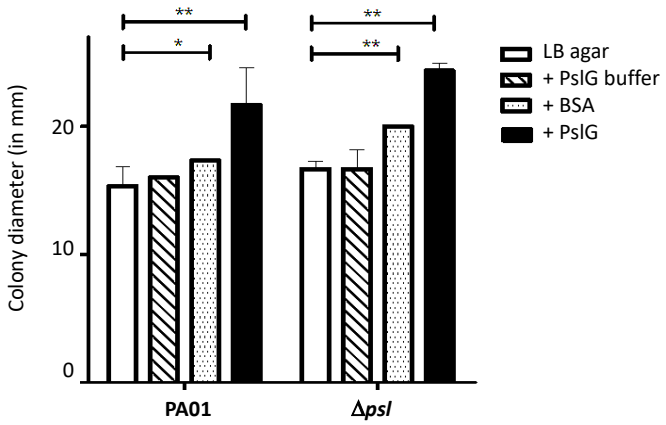


Figure 15. Effect of PslG and BSA supplementation on the motility of Psl-producing and Psl-deficient *P. aeruginosa.* Bacterial strains were point-inoculated on a nutritive agar plate with or without 0.1 mg/mL PslG or BSA -. Bacterial spreading was quantified by measuring the diameter of the resulting colonial growth. * and ** indicate a significant difference with the untreated group with respectively $p<0.05$ and $p<0.01$ by ANOVA.

Taken together, results of these experiments suggest that GH-media augmentation in motility is independent of GH catalytic activity and may be a generalized bacterial response to extracellular proteins.

3- Buffer effect.

Rationale: In previous experiments, we observed that treatment of mice with the PelA buffer alone was associated with much higher pulmonary bacterial burden and an increased rate of hematogenous dissemination as compared with the PslG buffer (as shown in previous reports and further, in Figure 17). The composition of PelA and PslG buffers differs primarily in that the PelA buffer contains a higher glycerol content. Glycerol can serve as a nutrient support for lung colonization by *Pseudomonas* (Sun et al. 2014 https://doi.org/10.1371/journal.pone.0103778; Scoffield et al. 2016 Can J Microbiol. 2016 doi: 10.1139/cjm-2016-0119). Therefore, we investigated the possibility for using PelA (and Ega3) in PslG buffer.

Methodology: First, we measured the melting temperature (Tm) which is the temperature at which the protein denatures and can be used as an indicator of the stability of the protein. The Tm of Ega3 and PelA in various buffers was determined using the Sypro orange dye assay on a qPCR machine to compare the fluorescence of the protein as the temperature increases. Protein in its original

buffer was used as a control while the buffers alone were used as blanks. Second, we assayed the enzymatic activity of GHs in the same buffers.

Results: The change of buffer did not significantly change the Tm of PelA and Ega3 (Table 2). Similarly, the enzymatic activity was not affected by the change of buffer (Figure 16).

GH	Average Tm	
	Original buffer	PslG buffer
PelA	51.82 °C	48.66 °C
Ega3	40.23 °C	42.44 °C

Table 2. Impact of the change of buffer on the melting temperature of GHs. The Tm is the temperature at which half of the enzymatic activity is lost.

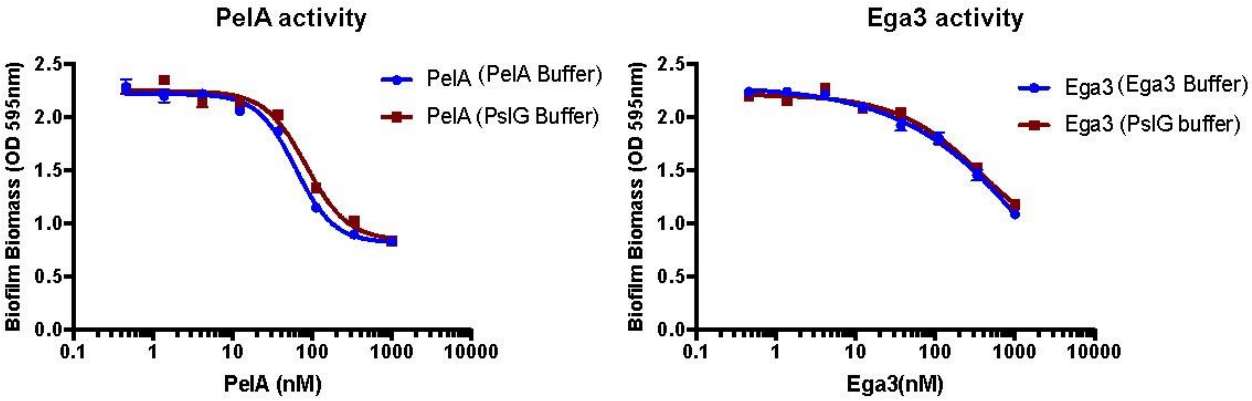


Figure 16: Enzymatic activity of PelA and Ega3 when re-suspended in various buffers, as measured by *P. aeruginosa* PA14 biofilm disruption assay.

The change of buffer should therefore not be a problem in terms of enzyme stability or activity. Thus, from now on, at the last step of production (All Major Tasks, Subtask 1), PelA and Ega3 will be eluted in PslG buffer in order to remove glycerol from the solution and any downstream potential complications that this additive may cause.

4- Effects of PslG and PelA therapies on the virulence of wild-type and motility-deficient, pilus-deficient *P. aeruginosa* strains *in vivo*.

Methodology: Mice were treated intratracheally with a 50 µL suspension containing $1.5x10^7$ bacteria (*P. aeruginosa* PAO1, or pilus-deficient *P. aeruginosa*), combined with 500 µg PslG, or PelA, or the sterile GH buffer, as indicated. At 48 hours post-infection, all mice were sacrificed and blood and lungs were harvested and plated for quantitative culture.

Results: Unlike previous experiments, PslG treatment did not result in increased pulmonary bacterial burden or hematogenous dissemination of wild-type *P. aeruginosa* (Figure 17). In contrast, while PslG treatment did not increase pulmonary bacterial burden of the pilus-deficient strain of *P. aeruginosa*, increased hematogenous dissemination was observed, suggesting that bacterial motility is dispensable for GH-mediated hematogenous dissemination.

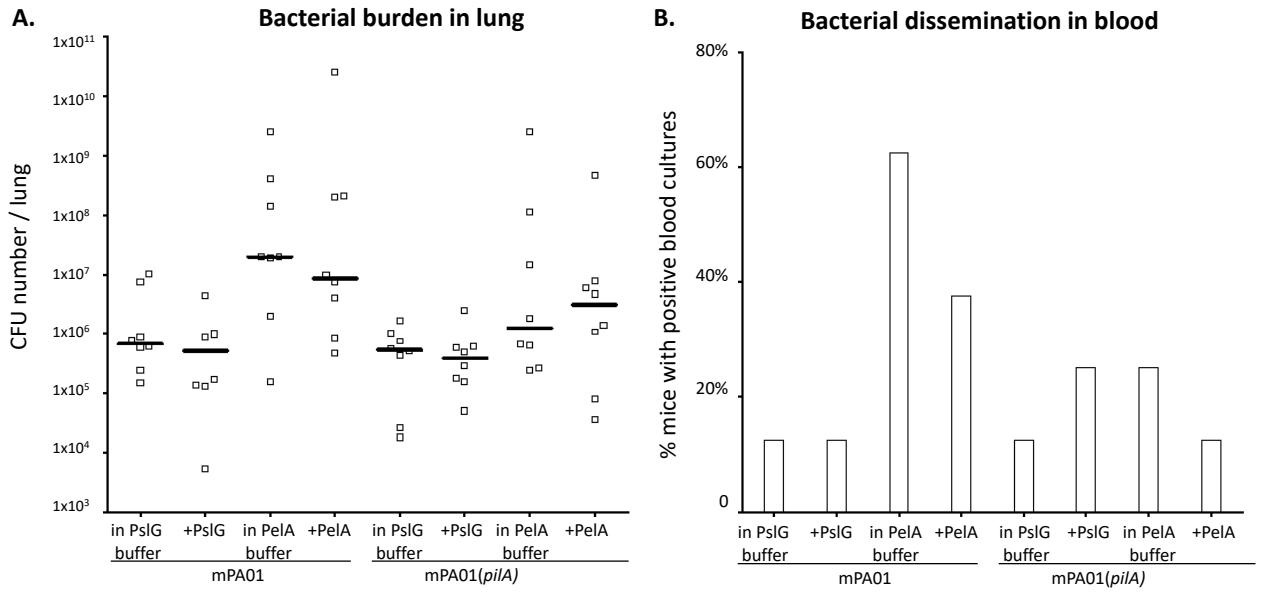


Figure 17. Effect of PslG and PelA on (A.) pulmonary bacterial burden and (B.) hematogenous dissemination of bacteria as determined by quantitative culture of lung homogenate and blood, respectively. Results shown are from a single experiment with 8 mice per condition. Horizontal lines indicated the median of each experimental group

In conclusion, the results of these experiments suggest that GH therapy alone does not result in significant improvement in the natural history of acute *P. aeruginosa* infection, and may instead enhance bacterial replication and dissemination. As such, we elected to focus our future studies on GH-antibiotic combination therapy as outlined in Major Task 7. This subtask is now complete.

☑ **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 hydrolases (Ega3) on fungal burden of immunocompetent mice chronically infected with *A. fumigatus*. [10 mice per group X 6 experimental groups X 1 hydrolase regimens X 2 time points AND 3 mice for histopathology at a single time point X 6 groups X 1 hydrolase regimens performed in duplicate = 276 mice] Dr Sheppard's lab. Months 13-30. Completion level = 100%.

Previous accomplishments:

During 2017-18 annual report, the first experiment with Sph3 and Ega3 was reported. We observed a trend of lower fungal burden in the group treated with Sph3, while an increase was observed in the group treated with Ega3. Neither result was statistically significant.

Accomplishments:

Methodology: A 50 μL suspension of agarose beads with or without 1.25×10^6 conidia of *A. fumigatus* strain Af293 embedded within them were administered intratracheally to immunocompetent female BALB/c mice. Beads were suspended in a solution containing 500 μg of

the appropriate GH, or sterile GH buffer. At days 1 and 7, mice were sacrificed and their lungs were harvested and homogenized for fungal burden quantification as assayed by galactomannan (GM) analysis (Platelia™ Aspergillus EIA, BioRad). GM readings were normalized to the weight of the harvested lung and to a highly infected lung homogenate standard. Lungs were also processed for histopathology examination to determine inflammatory responses and for exopolysaccharide immunostaining. Experiments were performed on two separate occasions, on groups of 8 mice per condition.

Results: The effects of intra-tracheal Ega3, Sph3 and PelA treatment in the mouse model of chronic aspergillus airway infection were tested in duplicate (Figure 18). When these results were combined, no significant change in fungal burden was observed following GH therapy, suggesting that GH therapy is not effective in the bead model of chronic pulmonary *Aspergillus* infection.

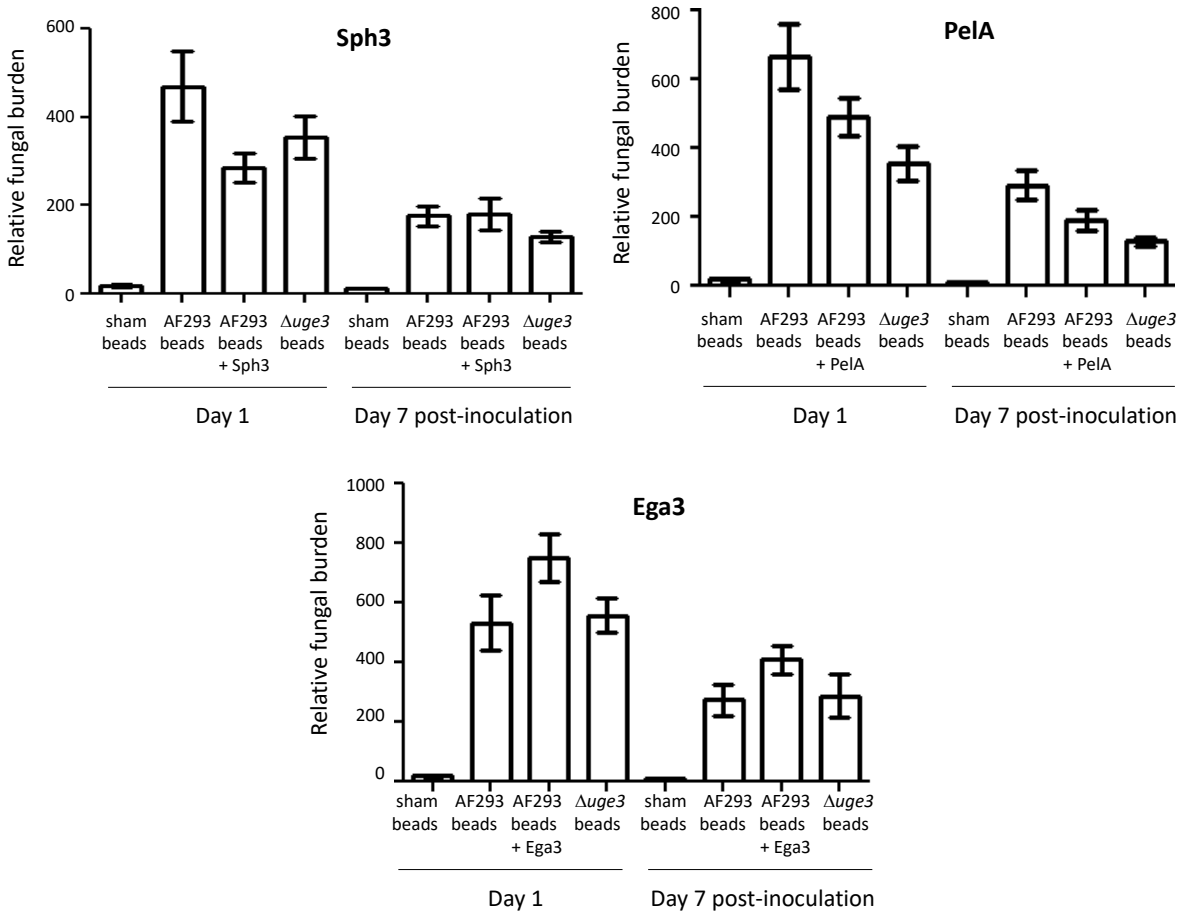


Figure 18: Relative fungal burden in lungs of immunocompetent mice intratracheally infected with a single dose of agar beads containing buffer only (sham) or 1.25x106 conidia of Af293, suspended in sterile buffer with or without 500 of the indicated GH. Fungal burden was determined by galactomannan content quantification (Platelia® Aspergillus EIA) of lung homogenates.

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*. [10 mice per group X 6 experimental groups X 2 hydrolase regimens X 3 strains X 2 time points AND 3 mice for histopathology X 6 groups X 2 hydrolase regimens X 3 strains at a single time point all performed in duplicate = 1656 mice] Dr Sheppard's lab. Months 18-30. Completion level = 25%.

Accomplishments:

1- Dose finding.

Methodology: Immunocompetent female BALB/c mice were infected intratracheally with a 50 µL suspension containing $1x10^6$, $2x10^6$ or $3x10^6$ beads of *P. aeruginosa*-containing agar beads. 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). At days 2 and 7 post-injection, mice were sacrificed and their lungs were harvested, homogenized and quantitative culture was performed. Lungs were also processed for histopathology studies to examine the inflammatory responses and for exopolysaccharide immunostaining.

Results: Dose finding studies to identify the optimal *P. aeruginosa* bead inoculum have been performed (Figure 19). High dose *P. aeruginosa* bead infection ($3x10^6$ organisms per mouse) resulted in an initially high pulmonary bacterial burden that rapidly declined by 7 days after infection, and was associated with mortality (2/15 mice). Further experiments were therefore not performed with this inoculation dose. Low dose infection (1×10^6 organisms/mouse) resulted in stable, low level infection in the majority of mice, but infection was not established in 100% of animals. Infection with 2×10^6 organisms/mouse produced reliable levels of infection with a slow decrease in bacterial burden over seven days. Future experiments evaluating the activity of GH therapy will therefore use this 2×10^6 dose.

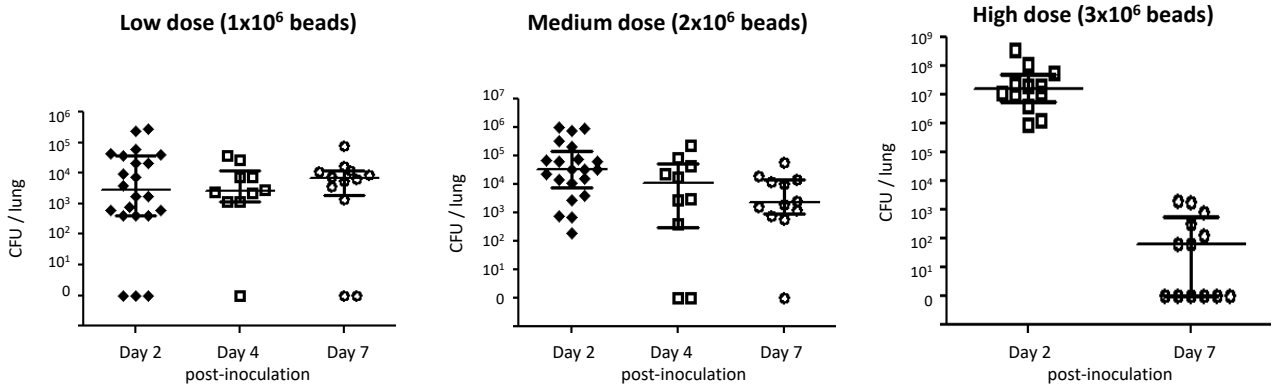


Figure 19. Pulmonary bacterial burden as determined by quantitative culture of lung homogenates. Horizontal lines indicate the median of each experimental group, error bars represent the 75th- and 25th-percentile. The experiment has been performed with groups of a minimum of 6 mice per condition. Low and medium doses were tested in triplicate, and high dose in duplicate.

2- Effect of hydrolases on the natural history of *P. aeruginosa* chronic infection.

Methodology: Mice were infected with *P. aeruginosa* beads as outlined above, and treated intratracheally with a combination of 250 µg of PslG and 250 µg PelA at the time of infection.

Results: GH treatment of mice infected with *P. aeruginosa* PA01 beads did not result in a significant reduction of bacterial burden at day 7 (Figure 20). This experiment will be repeated in the coming grant period).

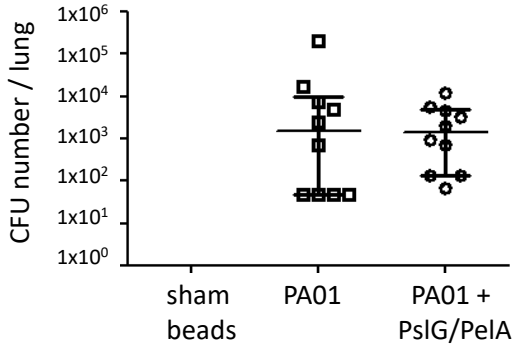


Figure 20. Pulmonary bacterial burden as determined by quantitative culture of lung homogenates, in mice infected by sterile agar beads, and *P. aeruginosa*-containing agar beads with and without PslG/PelA at 250 µg each. Horizontal lines indicate the median of each experimental group, error bars represent the 75th- and 25th-percentile. The experiment was performed once, with groups of a minimum of 6 mice per condition. No significant change was observed between groups with or without GH, using the Mann-Whitney test.

❑ **Milestone to achieve: To demonstrate 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 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. Completion level = 100%.

Rationale:

Our *in vitro* assays for potentiation of antifungal drugs by GHs (Major Task 1) previously 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), we 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 at two different antifungal concentrations at a single time-point.

Accomplishments:

Methodology: For the severely immunosuppressed mouse model (leukopenic model), mice were treated by subcutaneous injection of 250 mg/kg of cortisone plus intraperitoneal injection of 250 mg/kg of cyclophosphamide at day -2, followed by injection of 200 mg/kg cyclophosphamide and 250mg /kg of cortisone intraperitoneally at day +3. For the less acute immunosuppressed mouse model (neutrophil-depleted model), mice were treated with an antibody targeting the neutrophil-specific surface molecule Ly6G, by intraperitoneal injection of 200 µg of the antibody every 48h, beginning 1 day prior to infection.

At day 0, mice from both models were endotracheally infected with a 50 µL suspension containing 0 or 500 µg of Sph3 or PelA and 0 or $5x10^6$ conidia of Af293 *A. fumigatus*. Mice were then treated every 12 hours with the indicated dose of posaconazole by oral gavage and monitored for health signs. For fungal burden, the lungs were harvested and homogenized in PBS. Lung homogenates were assayed by galactomannan assay using the Platelia® Aspergillus EIA (BioRad); the GM values were then normalized to a highly infected lung homogenate standard.

Results:

1- Antifungal dose determination.

Dose-ranging studies were performed to determine an appropriate sub-therapeutic antifungal dose. In the leukopenic model (cortisone + cyclophosphamide), no effect of the posaconazole on the fungal burden could be observed (Figure 21).

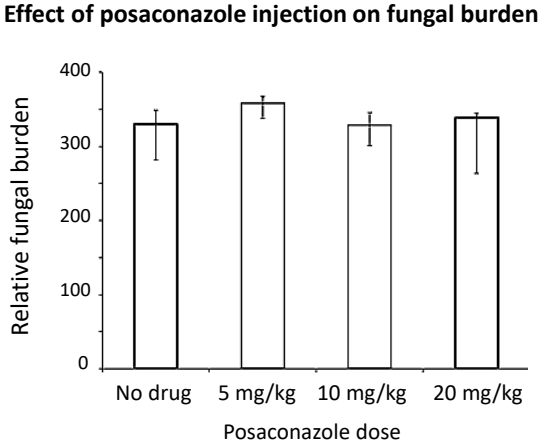


Figure 21: Fungal burden at day +4 in lungs of immunosuppressed mice intratracheally infected with $5x10^3$ conidia of *A. fumigatus* and treated orally every 12 hours with the indicated dose of posaconazole.

We therefore decided to move to the neutrophil-depleted model (Ly6G) and monitored survival as a therapeutic endpoint. In this model, 5 mg of posaconazole/kg of mice produced a significant but not maximal therapeutic effect (Figure 22). Posaconazole doses of 5 mg/kg and 2.5mg/kg were therefore selected for further study in combination with GH therapy.

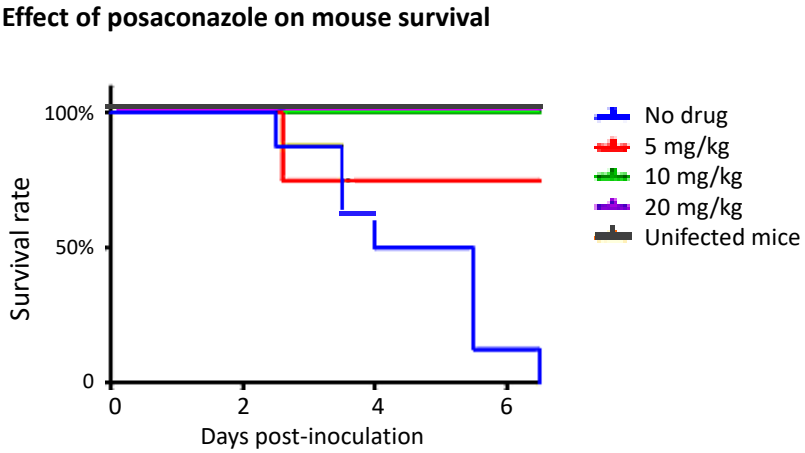


Figure 22. Survival of neutropenic mice infected with a single dose of *A. fumigatus* strain Af293 conidia (5x106) and treated orally with posaconazole every 12 hours. 8 mice per group in a single experiment.

2- <u>Potentiation assay of posaconazole by Sph3 and PelA</u>.

As was observed in our dose-finding studies, posaconazole monotherapy at 5 or 2.5mg/kg/day had no significant effect on pulmonary fungal burden (Figure 23). The effects of Sph3 monotherapy alone at 2 days of infection in the neutrophil depletion model were less marked than the one observed in our previous studies at 4 days of infection in the cortisone acetate-cyclophosphamide model (Major Task 5 Subtask 3). The addition of Sph3 to 2.5 mg/kg posaconazole resulted in a trend to reduced fungal pulmonary fungal burden as compared with posaconazole alone or Sph3 therapy alone; however these differences failed to reach statistical significance. Similar, though less dramatic findings were observed with the 5 mg/kg dose of posaconazole, in part due to a greater antifungal effect of this dose of posaconazole monotherapy.

The addition of PelA alone resulted in a dramatic reduction in pulmonary fungal burden; this effect precluded the assessment of PelA - posaconazole synergy. Although beyond the scope of the SOW, the effects of reduced dose PelA on the activity of posaconazole will be evaluated in the future.

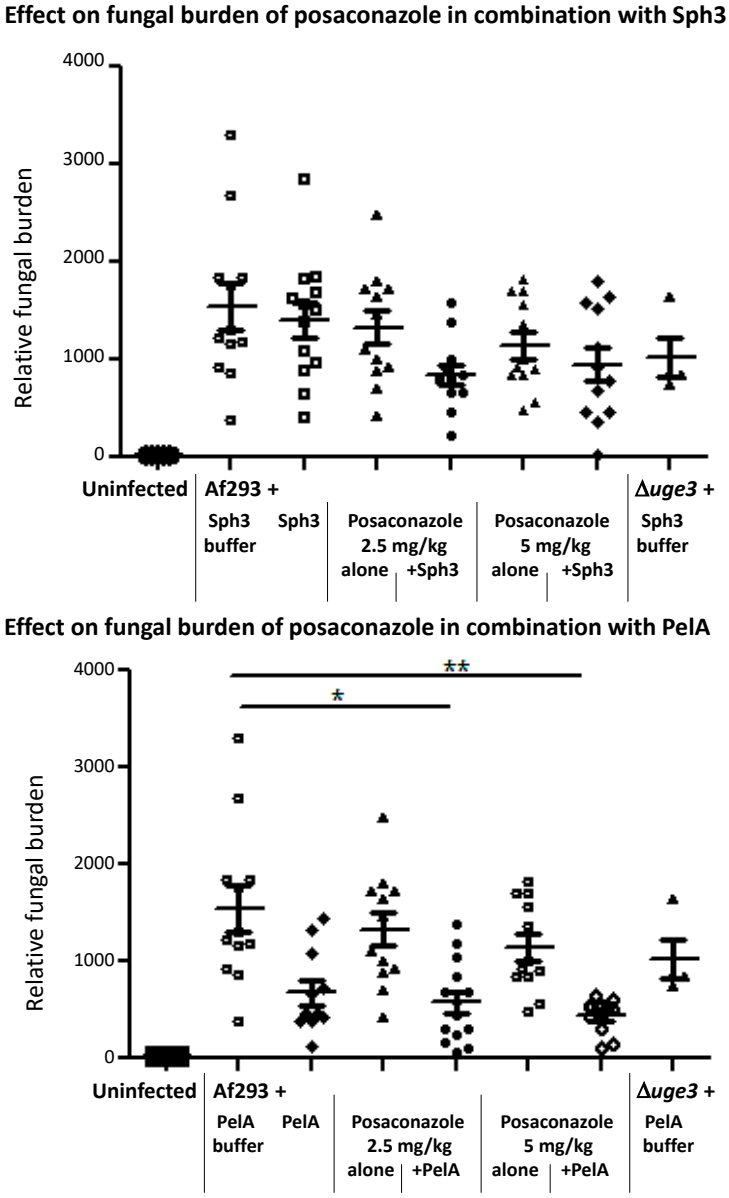


Figure 23. Pulmonary fungal burden of immunosuppressed mice intratracheally inoculated with $5x10^3$ conidia of the indicated *A. fumigatus* strain with or without 500 µg of the indicated GH, and then treated with the indicated dose of posaconazole every 12h for 2 days. Fungal burden was determined by galactomannan quantification (Platelia Aspergillus EIA) of lung homogenates. The experiment was performed with groups of 8 mice per condition, on 2 separate occasions (n=2). * and ** indicate a significant difference in GM content as compared with untreated, infected mice with respectively $p<0.05$ and <0.01 by ANOVA.

As per SOW, **this task is now completed**. However during the grant extension time we will re-test the potentiation of posaconazole with a reduced dose of PelA, and, if time allows, evaluate the effects of Sph3 and PelA in combination with caspofungin.

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 = 50%.

Previous accomplishments:

A dose of ciprofloxacin of 10mg/kg was determined to be the minimal dose required to result in a detectable reduction in pulmonary bacterial burden in our mouse model of pulmonary *Pseudomonas* infection. Building on these results, we evaluated the effects of combining GH therapy with antibiotics *in vivo* using the combination of ciprofloxacin with PslG or PelA. During previous report year, we performed 3 replicates of ciprofloxacin potentiation by PslG and 1 assay of ciprofloxacin potentiation by PelA. Initial results indicated a trend towards GH potentiation of ciprofloxacin but failed to reach statistical significance.

Accomplishments:

Methodology: Mice were intratracheally infected with a 50 µL suspension containing $1.5x10^7$ bacteria (*P. aeruginosa* PAO1), in combination with 500 µg of single GH, or 250 µg of each GH if in combination, or sterile GH buffer. Ciprofloxacin 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.

Results:

1- GH monotherapy did not significantly potentiate the effect of ciprofloxacin

PslG: The combined results of five experiments testing the ability of PslG to potentiate the action of ciprofloxacin failed to demonstrate a significant potentiation of ciprofloxacin by PslG (Figure 24). While ciprofloxacin treatment prevented PslG-mediated hematogenous dissemination of *P. aeruginosa*, the combination of PslG and ciprofloxacin was not superior to ciprofloxacin alone in reducing the pulmonary bacterial burden of infected mice.

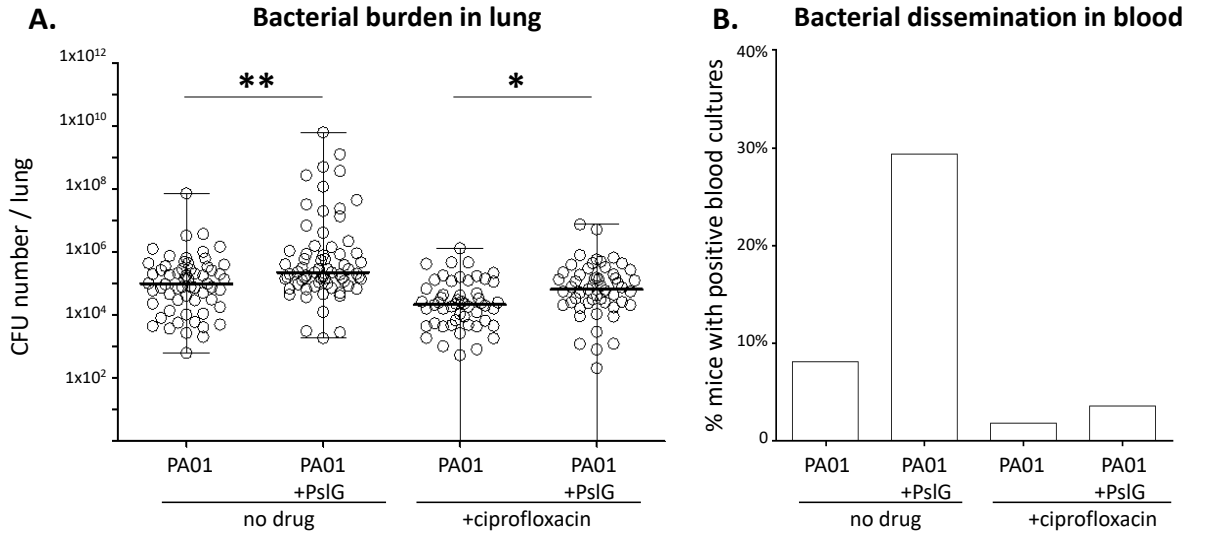


Figure 24. A. Effects of PslG and ciprofloxacin on the pulmonary bacterial burden as determined by quantitative culture of lung homogenate on LB plates. Horizontal lines indicate the median of each experimental group, error bars show the 75- and 25-percentile. B. Percentage of mice with positive blood cultures as determined by quantitative culture. The experiment has been performed with groups of 8 mice per condition, on 5 separate occasions (n=5). * and ** indicate a significant difference between untreated mice and those receiving PslG, with $p<0.05$ and <0.01 respectively, by Kruskall-Wallis test.

PelA: As with PslG monotherapy, PelA monotherapy did not potentiate the activity of ciprofloxacin (Figure 25).

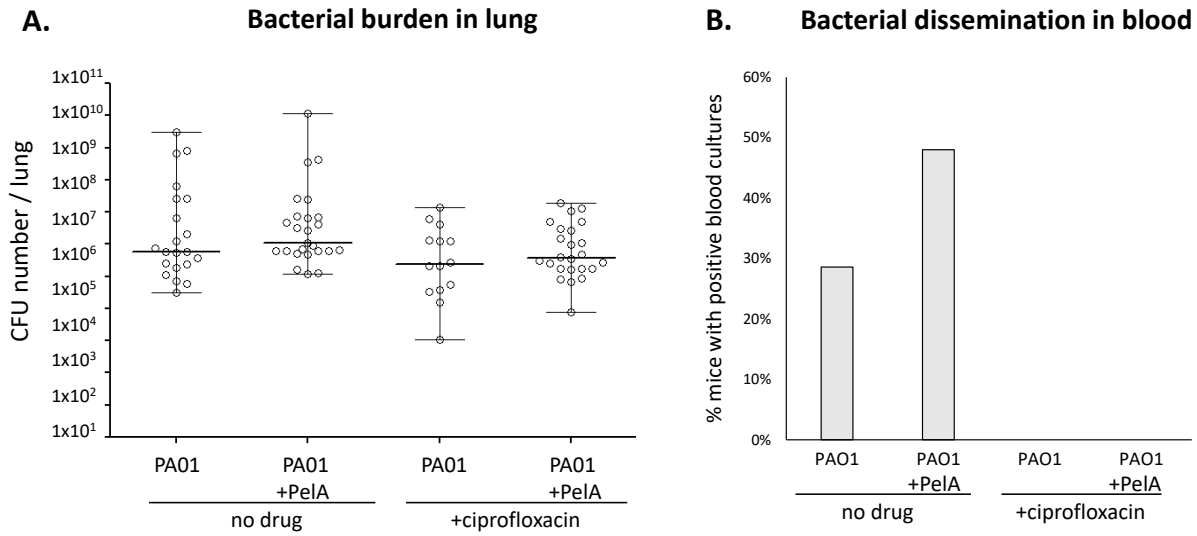


Figure 25. A. Effects of PelA and ciprofloxacin on the pulmonary bacterial burden as determined by quantitative culture of lung homogenate on LB plates. Horizontal lines indicate the median of each experimental group, error bars show the 75- and 25-percentile. B. Percentage of mice with positive blood cultures as determined by quantitative culture. The experiment has been performed with groups of 8 mice per condition, on 3 separate occasions (n=3). There was no significant difference between untreated mice and those receiving PelA, by Kruskall-Wallis test.

Ega3: As with other GH therapy, Ega3 monotherapy did not potentiate ciprofloxacin activity (Figure 26). In contrast to PslG and PelA however, Ega3 monotherapy did not result in increased hematogenous dissemination of bacteria.

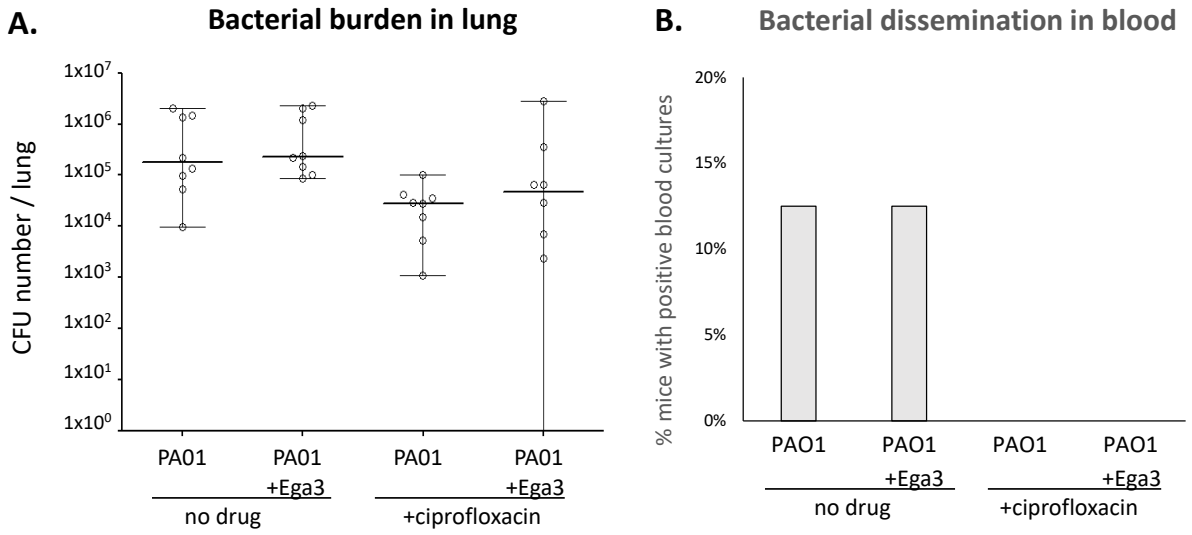


Figure 26. A. Effects of Ega3 and ciprofloxacin on the pulmonary bacterial burden of *P. aeruginosa* infected mice as determined by quantitative culture of lung homogenate on LB plates. B. Percentage of mice with positive blood cultures as determined by quantitative culture. Horizontal lines indicate the median of each experimental group, error bars show the 75- and 25-percentile. The experiment has been performed once with groups of 8 mice per condition (n=1). There was no significant difference between untreated mice and those receiving Ega3, by Kruskall-Wallis test.

Collectively these data suggest that therapy with a single GH does not potentiate ciprofloxacin. As *P. aeruginosa* can produce both Pel and Psl, it is possible that degrading both polymers is required to augment antibiotic efficacy *in vivo*. We therefore turned to the evaluation of combination GH therapy (PslG/Ega3 and PslG/PelA) to potentiate ciprofloxacin.

2- PslG/PelA combination therapy potentiates the effect of ciprofloxacin

The addition of PslG/PelA to ciprofloxacin was associated with a reduction in pulmonary bacterial burden as compared with ciprofloxacin or PslG/PelA therapy alone (Figure 27). As observed previously, GH treatment alone was associated with an increased rate of bacteremia, however this was not seen in ciprofloxacin-treated mice.

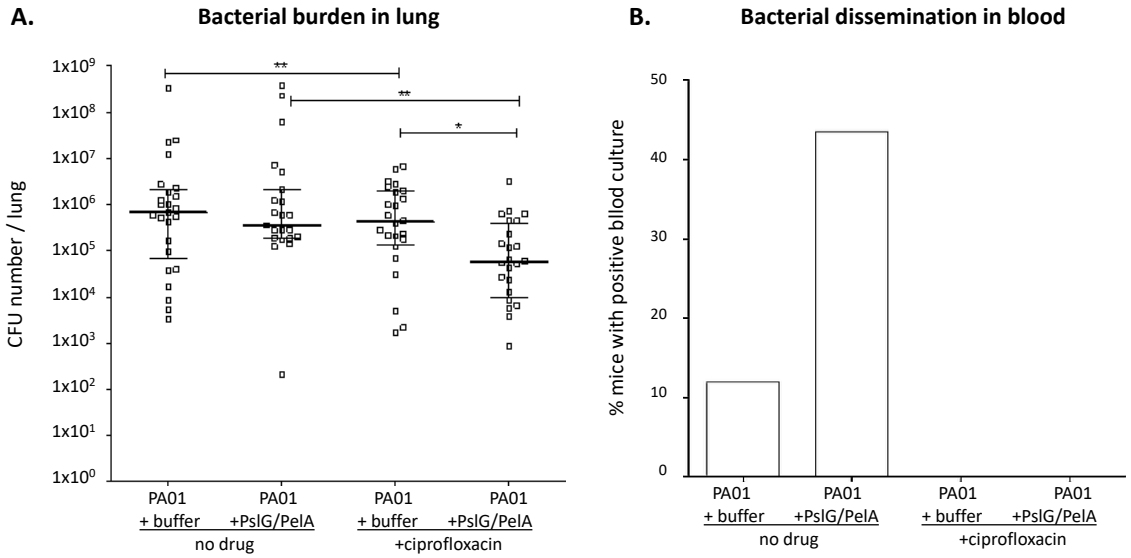


Figure 27. A. Effects of PslG/PelA and ciprofloxacin on the pulmonary bacterial burden of *P. aeruginosa* infected mice as determined by quantitative culture. B. Percentage of mice with positive blood cultures as determined by quantitative culture. Horizontal lines indicate the median of each experimental group, error bars show the 75th- and 25th-percentile. The experiment has been performed in triplicate with groups of 8 mice per condition. * and ** indicate a significant difference in pulmonary bacterial burden between indicated groups with respectively p<0.05 and <0.01 by Kruskall-Wallis test.

3- PslG/Ega3 combination therapy does not significantly potentiate the effect of ciprofloxacin

The addition of PslG/Ega3 to ciprofloxacin was associated with a trend to reduced pulmonary bacterial burden as compared with ciprofloxacin alone, but this was not statistically significant (Figure 28). As with PslG/PelA, GH therapy alone resulted in increased bacteremia, but the use of ciprofloxacin protected mice from this effect.

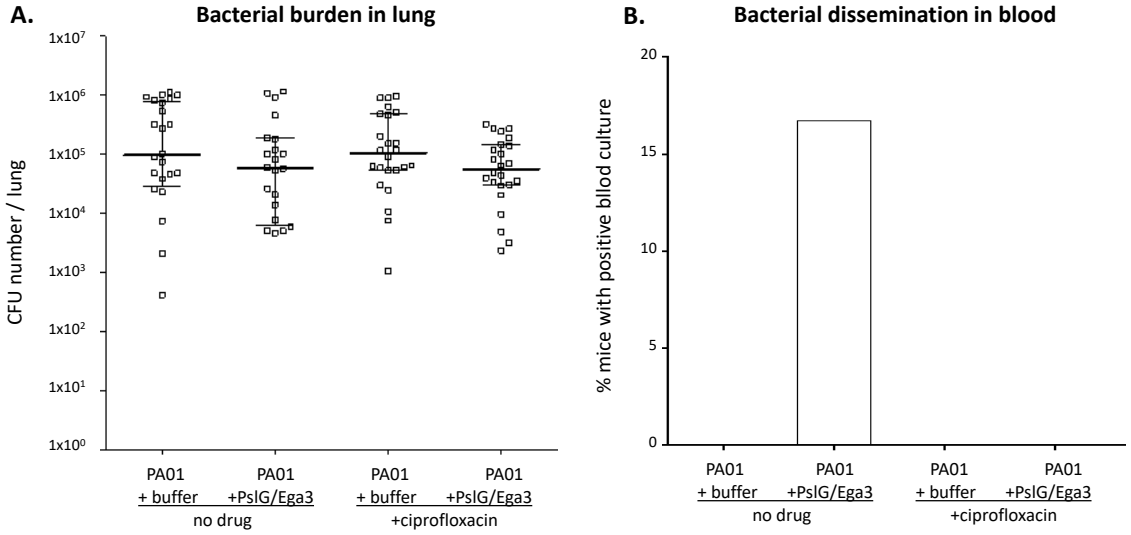


Figure 28. A. Effects of PslG/Ega3 and ciprofloxacin on the pulmonary bacterial burden of *P. aeruginosa* infected mice as determined by quantitative culture. B. Percentage of mice with positive blood cultures as determined by quantitative culture. Horizontal lines indicate the median of each experimental group, error bars show the 75th- and 25th-percentile. The experiment has been performed in triplicate with groups of 8 mice per condition. * and ** indicate a significant difference in pulmonary bacterial burden between indicated groups with respectively p<0.05 and <0.01 by Kruskall-Wallis test.

4- Determination of ceftazidime dose for acute model of mouse infection by *P. aeruginosa*.

Rationale: As with our other GH-antibiotic potentiation experiments, we first performed dose-ranging experiments to identify a dose of ceftazidime with a minimal therapeutic effect on pulmonary bacterial burden.

Methodology: Mice were intratracheally infected with a 50 µL suspension containing $1.5x10^7$ bacteria (*P. aeruginosa* PAO1). A range of doses of ceftazidime were administered intraperitoneally or subcutaneously at 4h, 12h and 20h post-infection. At 48 hours post infection, all mice were sacrificed. Lungs were harvested quantitatively cultured.

Results: A dose of 25 mg/kg of mouse injected subcutaneously produced a reproducible minimal reduction in bacterial burden, while doses of 50mg/kg and higher resulted in a dramatic reduction in bacterial burden (Figure 29). We therefore selected a dose of 25mg/kg for testing in combination with GH therapy to ensure that a potentiation of antimicrobial activity would be detectable.

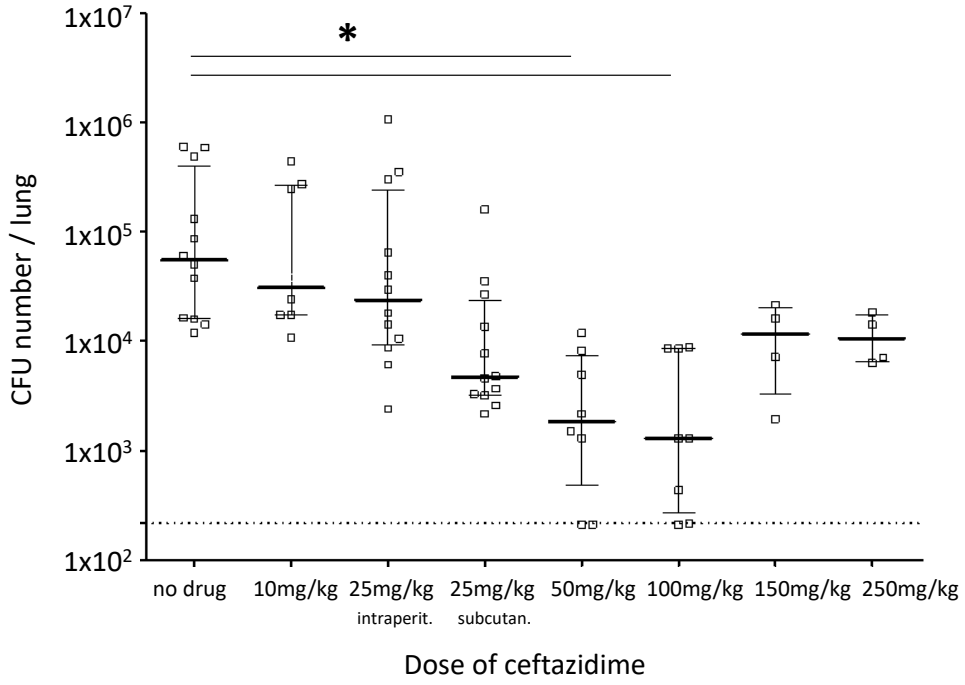


Figure 29. Effect of the indicated doses of ceftazidime on pulmonary bacterial burden as determined by quantitative culture. Ceftazidime was administered at 4h, 12h and 20h post infection by intraperitoneal injection (intraperit) or subcutaneously (subcutan) as indicated . Horizontal lines indicate the median of each experimental group, error bars indicate the 75th- and 25th-percentile. The experiment was performed in triplicate with groups of 6 mice per condition. * indicates a significant difference in pulmonary bacterial burden between indicated groups, $p<0.05$ 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?

Rachel Corsini and James Stewart were trained by Melanie Lehoux in regard to animal care. They learned to perform intratracheal injection of GHs in mice, as well as mouse infection with pathogens. They also learned to isolate *P. aeruginosa* from lung tissues and monitor this population.

Piyanka Sivarajah was trained by Ira Lacdao in modern biochemical techniques, specifically protein expression and purification.

François LeMauff presented his results in relation to this grant at the Canadian Glycomics symposium, Banff (Alberta), May 15-17, 2019.

How were the results disseminated to communities of interest?

Nothing to report

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 propose to complete the evaluation of the pulmonary tolerability and pharmacokinetics of this GH variant, and test the efficacy of intra-tracheal BCE5582 therapy in the mouse model of invasive aspergillosis as we have done with other GH enzymes.

Major Task 7: Test hydrolases for synergy with antimicrobials. The GH-antimicrobial combination studies which we have already initiated will be completed. These include: the effects of reduced dose PelA in combination with posaconazole, and the effects of Sph3 and PelA in combination with caspofungin in the mouse model of invasive aspergillosis; the effects of PslG/PelA and PslG/Ega3 in combination with ceftazidime in the acute model of *P. aeruginosa* pulmonary infection; and the effects of PslG/PelA and PslG/Ega3 in combination with ciprofloxacin in the chronic model of *P. aeruginosa* infection.

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

Two challenges were identified during the course of the grant as detailed below.

1- The Ega3 hydrolase was initially produced in the yeast *Pichia pastoris*. Intra-tracheal therapy with this glycoside hydrolase (GH) variant was associated with pulmonary eosinophil recruitment, suggesting that fungal-type glycosylation of the protein might induce allergic-type host responses. We therefore optimized the production of Ega3 in human HEK-293 cells, and confirmed that HEK-derived Ega3 did not induce pulmonary eosinophil recruitment. While this issue is resolved, this change in Ega3 production resulted in a ~6-month delay in our workflow as Ega3 is required for the majority of our in vivo studies. This delay has necessitated a 6 month no-cost extension to our grant period.

2- The pulmonary half-life of some of the GH enzymes was relatively short (~3h), and it was observed that GH enzyme half-life was prolonged in leukopenic mice, suggesting that leukocyte proteases may contribute to the degradation of GH enzymes *in vivo*. The optional studies to identify candidate GH variants with enhanced stability as outlined in Major Task 4 were therefore activated. As detailed in the "Accomplishments" section – Major Task 4, we have identified a PelA orthologue (BCE5582), with enhanced protease resistance in vitro, and will complete the in vivo pharmacokinetic, tolerability and efficacy studies to fully evaluate this GH variant in the 6 month no-cost extension.

Almost all Major Tasks are complete. There is no deviation from the originally proposed studies described in the originally submitted Statement of Work and subsequent progress reports. All animal use protocols for this work are approved and up to date. We foresee completing the remaining portion of the study within the granted additional 6-month period.

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

Speth C, Rambach G, Lass-Flörl C, Howell PL, Sheppard DC. *Galactosaminogalactan (GAG) and its multiple roles in* Aspergillus *pathogenesis*. Virulence. 2019. doi: 10.1080/21505594.2019.1568174.

Ostapska H, Howell PL, Sheppard DC. *Deacetylated microbial biofilm exopolysaccharides: It pays to be positive.* PLOS Pathogens. 2019. doi: 10.1371/ journal.ppat.1007411

Bamford, NC.*, Le Mauff, F.*, Subramanian, AS., Yip, P., Millan, C., Zhang, Y., Zacharias, C., Forman, A., Nitz, M., Codee, JDC., Uson, I., Sheppard, DC., and Howell, PL. *Ega3 from the fungal pathogen* Aspergillus fumigatus *is an endo-alpha-1,4-galactosaminidase that disrupts microbial biofilms.* Journal of Biological Chemistry – **Editor's pick**. 2019. doi:10.1074/jbc.RA119.009910, 2019

Zacharias CA, Sheppard DC. *The role of* Aspergillus fumigatus *polysaccharides in host-pathogen interactions.* Curr Opin Microbiol. 2019. doi: 10.1016/j.mib.2019.04.006. Review

Le Mauff F, Bamford NC, Alnabelseya N, Zhang Y, Baker P, Robinson H, Codée JDC, Howell PL, Sheppard DC. *Molecular mechanism of* Aspergillus fumigatus *biofilm disruption by fungal and bacterial glycoside hydrolases*. Journal of Biochemical Chemistry. 2019. doi: 10.1074 /jbc.RA119.008511

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:

PL Howell, Keynote Speaker: *Microbial Biofilms: mechanisms to potential therapeutics*. Annual Tri-University Symposium, University Guelph, May 7, 2019.

F Le Mauff, DC Sheppard: *Galactosaminogalactan synthesis is mediated by the cooperative activity of two glycosyl transferases*. Canadian Glycomics symposium, Banff (Alberta), May 15-17, 2019.

- **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

1. 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?

Name:	*Donald Sheppard*
Project Role:	PI
Research Identifier (e.g. ORCID ID):	0000-0001-8877-880X
Nearest person month worked	3
Contribution to Project:	Responsible for the research performed at Research Institute of the McGill University.
Name:	*Melanie Lehoux*
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	12
Contribution to Project:	Co-responsible for the animal work including preparation of animal use protocols
Name:	*Rachel Corsini*
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	12
Contribution to Project:	Co-responsible for the animal work
Name:	*James Stewart*
Project Role:	Masters Candidate
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	12
Contribution to Project:	Responsible for the test of the GHs in checkerboard combinations with antifungals against *A. fumigatus* biofilms
Name:	*Fabrice Gravelat*
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	12
Contribution to Project:	Responsible for the coordination of work between the two partner laboratories

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.

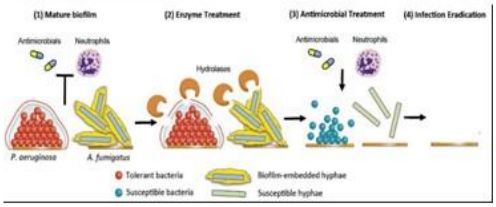
8. SPECIAL REPORTING REQUIREMENTS

AWARD CHART.

Award# W81XWH-16-1-0283 Log# PR150786: **Development of New Therapeutics Targeting Biofilm Formation by the Opportunistic Pulmonary Pathogens *Pseudomonas aeruginosa* and *Aspergillus Fumigatus*.**

PI: Dr Donald Sheppard, Research Institute of the McGill University Health Centre, Montreal (QC), Canada
Budget: $1,134,417.00 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 15-SEP-2019



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 and PEGylated GH is ongoing.
- ☑ 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 combination has been able so far to decrease microbial burden in lungs (studies ongoing).
- ☐ in invasive aspergillosis, Sph3 and PelA failed to potentiate the antifungal activity of posaconazole. In *P. aeruginosa* infection, PslG/PelA but not PslG/Ega3 potentiated the effect of ciprofloxacin. (other GH-antimicrobial combination studies ongoing)
- ☐ 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.

VIRULENCE
https://doi.org/10.1080/21505594.2019.1568174

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

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Galactosaminogalactan (GAG) and its multiple roles in *Aspergillus* pathogenesis

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ABSTRACT

Aspergillus spp and particularly the species *Aspergillus fumigatus* are the causative agents of invasive aspergillosis, a progressive necrotizing pneumonia that occurs in immunocompromised patients. The limited efficacy of currently available antifungals has led to interest in a better understanding of the molecular mechanisms underlying the pathogenesis of invasive aspergillosis in order to identify new therapeutic targets for this devastating disease. The *Aspergillus* exopolysaccharide galactosaminogalactan (GAG) plays an important role in the pathogenesis of experimental invasive aspergillosis. The present review article summarizes our current understanding of GAG composition and synthesis and the molecular mechanisms whereby GAG promotes virulence. Promising directions for future research and the prospect of GAG as both a therapy and therapeutic target are reviewed.

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Introduction

In order to cause pulmonary infection, microorganisms must both adhere to host cells, adapt to the natural environment imposed by the pulmonary environment and evade immune responses. One strategy used by the mold *Aspergillus fumigatus* to establish and maintain pulmonary infection is the production of biofilms during invasive infection in immunocompromised individuals and airway infection in patients with chronic lung disease [1]. Biofilms consist of stratified communities of organisms growing within a thick slime-like matrix of polysaccharides, proteins, lipids and nucleic acids that protect fungi from immune mediated killing and enhance resistance to antifungal agents [2,3]. Recent studies have established a key role for the exopolysaccharide galactosaminogalactan (GAG) in both the formation of *A. fumigatus* biofilms and in modulating the immune response during invasive infection.

GAG is a heteropolysaccharide composed of α-1,4 linked galactose, N-acetyl galactosamine (GalNAc) and galactosamine (GalN) [4–6] that is secreted by actively growing hyphae. GAG binds to the surface of these hyphae, resulting in a polysaccharide sheath that covers the growing organism and forms an extracellular matrix between hyphae [5]. GAG is expressed during chronic and invasive infection, and the production of cell wall GAG correlates with the intrinsic virulence of *Aspergillus* species [7]. Strains deficient in GAG do not form biofilms and are less virulent in mouse models of invasive aspergillosis (IA) [5]. Herein, we review our current understanding of the mechanisms underlying the synthesis of GAG, its role in the pathogenesis of invasive aspergillosis, and the current status of efforts to develop therapeutics targeting this important exopolysaccharide [5].

GAG biosynthesis

The biosynthetic pathway governing GAG production was identified by comparative transcriptional analyzes of *A. fumigatus* regulatory mutants deficient in the production of GAG [5]. This approach identified a cluster of five co-regulated genes on chromosome 3 which are predicted to encode enzymes with carbohydrate synthetic or modifying capacity [8]. Through gene disruption as well as structural and biochemical studies, a model of the function of these enzymes in GAG biosynthesis has begun to emerge (Figure 1). Synthesis of GAG begins with the conversion of UDP-glucose and UDP-N-acetyl glucosamine into UDP-galactose and UDP-N-acetyl galactosamine through the action of the cytosolic glucose-4 epimerase Uge3 [5,6]. Linking of these sugars, and export into the extracellular space is hypothesized to be mediated

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PEARLS

Deacetylated microbial biofilm exopolysaccharides: It pays to be positive

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Introduction

The production of biofilms is a common strategy used by many microorganisms during infection. Exopolysaccharides are a major component of the extracellular biofilm matrix serving to anchor organisms to surfaces, forming the structural scaffold of the biofilm, and protecting organisms from damage by hostile factors such as antibiotics and host immune defenses (Fig 1). Biochemical and genetic studies of biofilm exopolysaccharide synthesis have revealed that production of *N*-acetyl hexosamine-containing exopolysaccharides is one strategy used by diverse pathogens to facilitate biofilm formation and virulence. Following polymerization and extracellular extrusion by membrane embedded glycosyl transferases ($[HexNAc]_n$ + nucleotide-HexNac → $[HexNAc]_{n+1}$ + nucleotide), these glycans then undergo postsynthetic enzymatic deacetylation ($[HexNAc)_n$ → HexN-$[HexNAc]_{n-1}$ + acetyl group) to render them cationic. Deacetylation is critical for the function of these glycans in biofilm formation and host–pathogen interactions. This Pearl explores the role of these deacetylated cationic exopolysaccharides within the biofilm matrix in microbial pathogenesis and resistance to antimicrobial agents, and their potential as antibiofilm therapeutic targets.

Partially deacetylated, cationic hexosamine polymers are common in biofilm forming microorganisms

A wide range of medically important microbial species produce and secrete hexosamine-rich exopolysaccharides into their self-produced extracellular biofilm matrices (Table 1). The best studied example of these glycans is poly-β-1,6-*N*-acetylglucosamine (PNAG), a homopolymer of *N*-acetylglucosamine (GlcNAc) residues produced by a wide range of gram-positive and gram-negative pathogenic bacteria, including *Staphylococcus* spp., *Yersinia pestis*, *Bordetella* spp., and *Escherichia coli* [1–4]. The gram-negative opportunistic pathogen *Pseudomonas aeruginosa* produces several biofilm-associated exopolysaccharides, including the linear heteropolymer Pel, composed of GlcNAc and *N*-acetyl galactosamine (GalNAc), whereas the gram-positive organism *Listeria monocytogenes* produces a β-1,4-linked *N*-acetylmannosamine polysaccharide decorated with terminal α-1,6-linked galactose (Gal) residues [5,6]. More recently, biofilm formation by the opportunistic filamentous fungal pathogen *Aspergillus fumigatus* was found to be dependent on galactosaminogalactan (GAG), a heteropolymer composed of α-1,4-linked GalNAc and Gal residues [7].

Ega3 from the fungal pathogen *Aspergillus fumigatus* is an endo-α-1,4-galactosaminidase that disrupts microbial biofilms

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Aspergillus fumigatus **is an opportunistic fungal pathogen that causes both chronic and acute invasive infections. Galactosaminogalactan (GAG) is an integral component of the *A. fumigatus* biofilm matrix and a key virulence factor. GAG is a heterogeneous linear α-1,4–linked exopolysaccharide of galactose and GalNAc that is partially deacetylated after secretion. A cluster of five co-expressed genes has been linked to GAG biosynthesis and modification. One gene in this cluster, ega3, is annotated as encoding a putative α-1,4-galactosaminidase belonging to glycoside hydrolase family 114 (GH114). Herein, we show that recombinant Ega3 is an active glycoside hydrolase that disrupts GAG-dependent *A. fumigatus* and Pel polysaccharide-dependent *Pseudomonas aeruginosa* biofilms at nanomolar concentrations. Using MS and functional assays, we demonstrate that Ega3 is an endo-acting α-1,4-galactosaminidase whose activity depends on the conserved acidic residues, Asp-189 and Glu-247. X-ray crystallographic structural analysis of the apo Ega3 and an Ega3-galactosamine complex, at 1.76 and 2.09 Å resolutions, revealed a modified $(\beta/\alpha)_8$-fold with a deep electronegative cleft, which upon ligand binding is capped to form a tunnel. Our structural analysis coupled with *in silico* docking studies also uncovered the molecular determinants for galactosamine specificity and substrate binding at the −2 to +1 binding subsites. The findings in this study increase the structural and mechanistic understanding of the GH114 family, which has >600 members encoded by plant and opportunistic human pathogens, as well as in industrially used bacteria and fungi.**

Aspergillus fumigatus is a ubiquitous, filamentous fungus that causes invasive infections in immunocompromised patients (1). *A. fumigatus* can also cause chronic infections in patients with pre-existing lung conditions such as chronic obstructive pulmonary disease or cystic fibrosis (2–4). Even with currently available antifungal agents, the mortality of invasive aspergillosis remains over 50%, highlighting the need for new therapies that target *A. fumigatus* (2). During infection, *A. fumigatus* adopts a biofilm mode of growth, encapsulating itself in a self-produced matrix. The exopolysaccharide galactosaminogalactan (GAG)[7] is an integral component of the *A. fumigatus* matrix and a key virulence factor (5–9). GAG mediates fungal adhesion to host cells and inhibits the host immune response by masking fungal β-glucan from dectin-1 recognition and inducing neutrophil apoptosis and secretion of the immunosuppressive cytokine interleukin 1 receptor antagonist (5, 7, 10).

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[7] The abbreviations used are: GAG, galactosaminogalactan; PDB, Protein Data Bank; GH, glycoside hydrolase; PNAG, poly-β-1,6-GlcNAc; RMSD, root-mean-square deviation; SEC, size-exclusion chromatography; ACN, acetonitrile.

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Current Opinion in Microbiology

The role of *Aspergillus fumigatus* polysaccharides in host–pathogen interactions

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Aspergillus fumigatus is a saprophytic mold that can cause infection in patients with impaired immunity or chronic lung diseases. The polysaccharide-rich cell wall of this fungus is a key point of contact with the host immune system. The availability of purified cell wall polysaccharides and mutant strains deficient in the production of these glycans has revealed that these glycans play an important role in the pathogenesis of *A. fumigatus* infections. Herein, we review our current understanding of the key polysaccharides present within the *A. fumigatus* cell wall, and their interactions with host cells and secreted factors during infection.

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Introduction

The saprophytic mold *Aspergillus fumigatus* is found throughout the environment, where it plays an important role in decomposition and nutrient recycling [1]. *A. fumigatus* produces copious amounts of airborne conidia, which are easily dispersed by air currents. It has been estimated that the average human inhales hundreds of these conidia daily [1]. Both the relatively small size of *A. fumigatus* conidia (approximately 2 μm in diameter), as well as their strongly hydrophobic surface, enhance the ability of these particles to reach the terminal airways of the human host [1,2]. In healthy individuals, conidia are rapidly eliminated by the action of the mucociliary escalator or phagocytosed, and killed by resident alveolar macrophages and pulmonary epithelial cells [2,3]. Conidia that evade eradication by these mechanisms can swell and germinate, leading to the induction of a robust inflammatory response involving the recruitment and activation of neutrophils. These cells mediate the killing of germinating hyphae by the release of reactive oxygen species (ROS) and antimicrobial peptides [3,4]. However, in immunocompromised hosts or those with abnormal lung function, *A. fumigatus* hyphae can persist within the pulmonary system to establish an acute invasive or chronic airway infection, respectively [3]. In immunocompromised patients, such as those receiving cytotoxic chemotherapy, the innate immune response is unable to restrict fungal growth and hyphae that invade the lung parenchyma, causing tissue injury, and if unchecked, systemic dissemination [3]. Mortality rates for this disease can reach 90% in disseminated disease [5•]. In patients with chronic pulmonary disease, such as those with cystic fibrosis, the conidia are poorly cleared by the dysfunctional pulmonary mucociliary elevator. These conidia can then germinate and grow within the airways and pulmonary mucus layer. Hyphae remain largely contained to the airways due to the presence of a functional systemic immune system, although chronic, slowly progressive cavitary disease can develop [3,6]. Chronic pulmonary aspergillosis syndromes can lead to debilitating pulmonary and systemic inflammation as well as worsening of pulmonary function [6].

The *A. fumigatus* cell wall is a key point of contact between *A. fumigatus* and the host [7,8] (Figure 1). The majority of the fungal cell wall is composed of polysaccharides, and, as a result, there has been great interest in elucidating the role that these macromolecules play in host-fungal interactions. While recent studies with purified polysaccharides and mutant strains with altered polysaccharides have begun to shed light on the role of these molecules during infection, it is important to acknowledge the limitations of both experimental approaches. Purified polysaccharides may vary in size and composition from their native forms, and the immune response to soluble or microparticular polysaccharides may be different from the response to polysaccharides presented in their natural context where they immobilized within the cell wall and linked to other glycans and proteins. These effects have been best illustrated in studies of interactions with the cell wall polysaccharide chitin in which both particle size and the presence of co-stimulatory pattern recognition receptor ligands have a dramatic effect on the type of host response to this glycan (detailed below) [9,10••]. The use of synthetic oligosaccharides of defined length and composition may be helpful in this regard, but

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Molecular mechanism of *Aspergillus fumigatus* biofilm disruption by fungal and bacterial glycoside hydrolases

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ABSTRACT

During infection, the fungal pathogen *Aspergillus fumigatus* forms biofilms that enhance its resistance to antimicrobials and host defenses. An integral component of the biofilm matrix is galactosaminogalactan (GAG), a cationic polymer of α-1,4-linked galactose and partially deacetylated N-acetylgalactosamine (GalNAc). Recent studies have shown that recombinant hydrolase domains from Sph3, an *A. fumigatus* glycoside hydrolase involved in GAG synthesis, and PelA, a multi-functional protein from *Pseudomonas aeruginosa* involved in Pel polysaccharide biosynthesis, can degrade GAG, disrupt *A. fumigatus* biofilms, and attenuate fungal virulence in a mouse model of invasive aspergillosis. The molecular mechanisms by which these enzymes disrupt biofilms have not been defined. We hypothesized that the hydrolase domains of Sph3 and PelA ($Sph3_h$ and $PelA_h$, respectively) share structural and functional similarities given their ability to degrade GAG and disrupt *A. fumigatus* biofilms. MALDI-TOF enzymatic fingerprinting and NMR experiments revealed that both proteins are retaining endo-α-