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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

The bacterium *Pseudomonas aeurginosa* (PA) and fungus *Aspergillus fumigatus* (AF) are common causes of pulmonary disease in immunocompromised patients. During infection, both microorganism form a biofilm, which includes exopolysaccharides Pel and Psl (PA) or galactosaminogalactan (AF). PelA, PslG, Sph3 and Ega3 are recently discovered glycoside hydrolases that disrupt PA and AF biofilms *in vitro*. Our aim is to demonstrate that these enzymes can be used to impair biofilm formation in the host tissues as well, therefore attenuating the infection.

During this reported period, we have demonstrated that, *in vitro*, the enzymes impair the development of normal biofilm both in static culture and in a fluid biofilm culture system, and that they potentiate the action of antibiotics directed at PA or at AF. We have also demonstrated that, when injected intratracheally in mice, the hydrolases did not induce adverse immune reaction, nor did they induce any tissue damage to the host. Pharmacokinetics of the hydrolases in lungs is being assessed, first results showing that Ega3 and PslG persist longer than 12h and 72h, respectively, while Sph3 and PelA are degraded in under 12h.

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, $PelA_h$, and $PslG_h$ from PA and $Ega3_h$, and $Sph3_h$ 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:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

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

MAJOR GOALS FOR YEAR 1:

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 hydrolases.

Subtask 1: Express and purify $Sph3_h$, $Ega3_h$, $PelA_h$ and $PslG_h$ for subtasks 1-3 (Months 1-9). (PI: Howell)

Subtask 2: Test $Sph3_h$, $PelA_h$ and $Ega3_h$ in checkerboard combinations with antifungals against *A. fumigatus* biofilms (Months 1-9). (PI: Sheppard)

Subtask 3: Test $PslG_h$/$PelA_h$ and $PslG_h$/$Ega3_h$ in checkerboard combinations with antibiotics against *P. aeruginosa* biofilms (Months 1-9). (PI: Howell)

Subtask 4: Test candidate hydrolase-antimicrobial combinations in an in vitro fluid biofilm culture model system (Months 6-12). (PI: Howell and Sheppard)

Milestone: *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.

Major Task 2: Test candidate hydrolases for toxicity in vivo.

Subtask 1: Submit documents for Animal use approvals (Months 1-6). (PI: Sheppard)

Milestone*: obtain animal use approvals.*

Subtask 2: Express and purify recombinant $PelA_h$ and $PslG_h$ for subtasks 3 - 4 (Months 6-12). (PI: Howell)

Subtask 3: Test toxicity of pulmonary administration of hydrolase combinations ($PslG_h$/$PelA_h$ and $PslG_h$/$Ega3_h$ combinations) in immunocompetent mice [10 mice per group X 4 experimental groups X 2 hydrolase regimens; 1 group of 10 untreated mice. All performed in duplicate = 180 mice] (Months 6-12). (PI: Sheppard)

Subtask 4: Test toxicity of pulmonary administration of hydrolases ($PslG_h$/$PelA_h$ and $PslG_h$/$Ega3_h$ combinations) in immunocompromised mice [10 mice per group X 4 experimental groups X 2 hydrolase regimens; 1 group of 10 untreated mice. All performed in duplicate = 180 mice] (Months 6-12). (PI: Sheppard)

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

Major Task 3: Pharmacokinetic studies of candidate hydrolases

Subtask 1: Express and purify $Sph3_h$, $Ega3_h$, $PelA_h$ and $PslG_h$ for subtasks 2 - 5 (Months 6-12). (PI: Howell)

Subtask 2: Test pharmacokinetics of hydrolases ($Sph3_h$, $Ega3_h$, $PelA_h$ and $PslG_h$/$PelA_h$ and $PslG_h$ / $Ega3_h$ combinations) in immunocompetent 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] (Months 6-12). (PI: Sheppard)

Subtask 3: Test pharmacokinetics of hydrolases ($Sph3_h$, $Ega3_h$, $PelA_h$ and $PslG_h$/$PelA_h$ and $PslG_h$/ $Ega3_h$ combinations) in immunocompromised 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 (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.*

What was accomplished under these goals?

Accomplishments for Year 1:

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 hydrolases.

Subtask 1: Express and purify $Sph3_h$, $Ega3_h$, $PelA_h$ and $PslG_h$ for subtasks 1-3. Dr Howell's lab.
SOW Time period: Months 1-9. Completion level = 100%.

Background:
Before the initiation of the grant, our labs were able to produce the GHs $Sph3_h$, $PelA_h$ and $PslG_h$ in an *E. coli* expression system. This method reliably provides sufficient amounts of GH for *in vitro* experiments. However, *in vivo* experiments, as planned per SOW, require the production of substantial quantities of pure endotoxin-free protein. Therefore, we switched production from our standard *E. coli* system to either the endotoxin free *E. coli* bacterial system ClearColi® or to the *Pichia pastoris* yeast system PichiaPink®. To optimize the GH production we assayed multiple culture conditions.

Developments:

1- GH production in bacterial system.

Methodology:
In our original protocol the GH coding sequence was cloned into a vector that allows the proteins to be produced with a histidine tag; after cell growth the culture supernatant was harvested and

buffered at pH 8; finally, the GHs were purified using nickel affinity and size exclusion chromatography. The production of the $Sph3_h$, $PelA_h$ and $PslG_h$ GHs has been attempted and subsequently optimized in the bacterial system ClearColi®.

Optimization of $Sph3_h$, $PelA_h$ and $PslG_h$ expression:
Successful optimization efforts involved: growth in both richer, and autoinduction media; growth in Fernbach flask and higher shaking speeds to improve aeration of rich media.
These changes in protocol allowed an increase in the production of GHs up to 8 times, as compared to the original production. Final yields were 125 mg of $Sph3_h$ / liter of culture medium, and more than 80 mg of $PelA_h$, and $PslG_h$ (Table 1), which will be more than adequate for our needs for *in vivo* assays.

Protein	Media & Volume (L)	Protein Yield (mg)	Protein Yield (mg) / Media Volume (L)	Protein Yield (mg) / Cell weight (g)
Sph3	Luria Broth (LB), 3L	84mg	28	8
	LB, 2L	36mg	18	6.8
	Terrific Broth (TB), 1L, fernbach	125mg	125	19.3
PelA	LB, 4L	94.57mg	24	9.8
	LB, 2L	35.52mg	17.8	7.4
	TB , 1L, fernbach	84.15mg	84.2	17.4
PslG	LB, 4L	32.3mg	8	3.8
	TB, 2L	28.9mg	14.4	6.6
	Autoinduction, 0.5L, fernbach	41.48mg	83	10.5

Table 1. Optimization of GH production in *E. coli*. For each GH, the first line represents the original protocol; the following lines represent optimization progress.

While the optimization proved effective for $Sph3_h$, $PelA_h$ and $PslG_h$, we were unable to produce soluble $Ega3_h$ in *E. coli* and therefore optimization of this GH was performed in the PichiaPink® yeast system.

2- <u>$Ega3_h$ production in yeast system.</u>
Originally, we had proposed to move the production of $Sph3_h$, $PelA_h$ and $PslG_h$ to *P. pastoris*, and the first steps of this transfer have been successfully performed, as mentioned in "Technical Report of Year 1- Quarter 2". With the improvements to the *E. coli* ClearColi® production protocol (described above), we decided to keep $Sph3_h$, $PelA_h$ and $PslG_h$ production in the bacterial system. Only optimization of $Ega3_h$ production has been undertaken in *P. pastoris.*

Methodology:
The gene sequence was modified by codon optimization to maximize the protein expression, and cloned in pPinkα-HC plasmid (Invitrogen) under the control of the *AOX1* promoter for induction in the presence of methanol (Figure 1). A N-terminal alpha-factor secretion signal was also added to the protein. The construct was used to transform a *P. pastoris* strain which lacks the *ade2* gene; transformants able to grow on a medium without adenine were screened for integration of the vector in the yeast genome. In successful transformants, after translation, the N-terminal alpha-factor secretion signal allows $Ega3_h$ to be secreted into the yeast growth media. Culture

supernatant was therefore harvested, filtered and buffered at pH 8 and the protein purified using ammonium sulfate precipitation, followed by size exclusion chromatography.
This method yielded ~ 2.5 mg of Ega3$_h$ for every 1 L of growth.

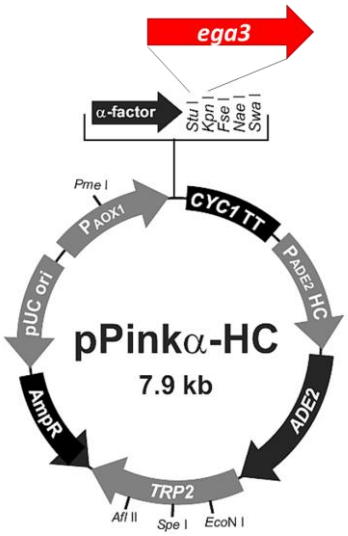


Figure 1. Genetic map of the vector used for expression of GHs in *P. pastoris*. The coding sequence of the desired GH (red) was cloned after the N-terminal alpha-factor secretion signal (α-factor). It is therefore placed under control of the *AOX1* promoter (P_{AOX1}) and *cyc1* terminator. A *ade2* cassette serves as a marker for the selection of successful transformants.

Optimization of Ega3$_h$ expression (Figure 2):
To optimized the expression we systematically explored: growth at higher shaking speed to improve aeration; stepwise expression: starvation of the cells; acclimation to methanol by addition of small amounts; addition of more methanol to start protein expression; addition of a second round of expression, transferring cells to new expression media; decreasing the pH of the media to pH6 to reduce degradation of Ega3$_h$ (data not shown).

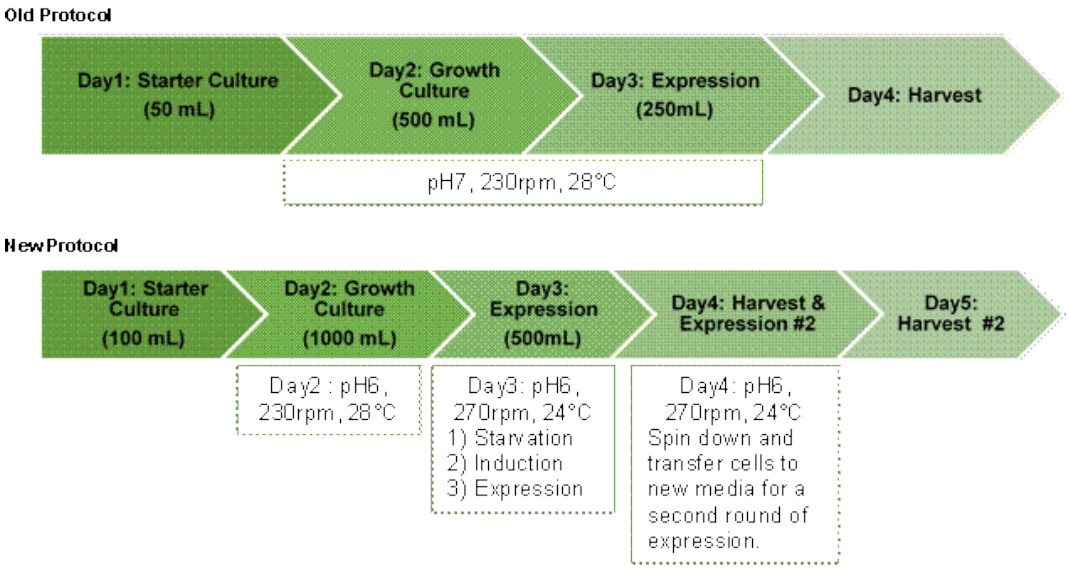


Figure 2. Schematic comparison of the old and new GH production methods in *P. pastoris*.

Results:
The improvements in protocol increased the yield of the protein by 4 fold to a final yield of 8 mg of protein / liter of culture medium (Table 2).

Protein	Media pH	Growth Volume (L)	Expression Volume (L)	Protein Yield (mg)	Protein Yield (mg) / Growth Volume (L)
$Ega3_h$	7	5.8L	2.9L	8.6mg	1.5
	7	6L	3L	10.3mg	1.7
	7	2L	2L	13.0mg	6.5
	6	4L	2L	31.6mg	7.9
	6	4L	4L	46.5 mg	11.6

Table 2. Optimization of GH production in *P. pastoris*. The first line represents our original protocol.

3- Control of GH quality: The activity of each enzyme is checked and quantified using our standard biofilm assays prior to their use in Subtasks 2-3. To assess whether there were any negative impacts to the enzyme as a consequence of the changes in the expression/purification protocol, we assayed the activity of the GHs using our biofilm disruption assay: The results revealed similar biofilm disruption activity throughout the protocol modifications (data not shown).

Subtask 2: Test $Sph3_h$, $PelA_h$ and $Ega3_h$ in checkerboard combinations with antifungals against *A. fumigatus* biofilms. Dr Sheppard's lab. SOW Time Period: Months 1-9. Completion level = 100%.

Background: Our preliminary data had reported that GH treatment enhanced the *in vitro* activity of the antifungal posaconazole against AF. We proposed to expand these studies to test other antifungal agents, and perform checkerboard studies to examine the effects of varying GH concentration on antifungal activity.

Accomplishments:

Methodology: Antifungal effects on AF were measured by quantifying metabolic activity using the well-described XTT assay. Briefly, *A. fumigatus* biofims were pre-grown at 37°C for 9 h in RPMI media in 24 well plates; after which a gradient of antifungal drug was added to the wells in the presence or absence of 0.5 μM of the indicated GH. Plates were incubated for an additional 15 h and fungal viability was then measured using the XTT metabolic assay. The MIC_{50} was defined as the minimum concentration of antifungal required to inhibit fungal metabolism by 50%.

Fungal inhibition was also scored visually in accordance with the guidelines stipulated by the Clinical and Laboratory Standards Institute (CLSI) Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi (Figure 3). In this assay, $1x10^4$ conidia of strain Af293 were added to 96-well plates containing the GH-antifungal combination of interest and incubated for 24 h at 37°C, 5% CO_2. Plates were then examined microscopically and the minimum inhibitory concentration required for a 90% reduction in fungal growth (MIC_{90}) was recorded. As per CLSI guidelines, for the echinocandin caspofungin, the minimum effective concentration (MEC) resulting in a significant change in morphology was used rather than growth inhibition. For each combination of antifungal/GH, at least 3 independent experiments were performed.

Originally, we had planned to study voriconazole as a representative of the azole class of antifungals; however, since its antifungal activity was not potentiated by GHs, it was replaced by posaconazole in all subsequent assays.

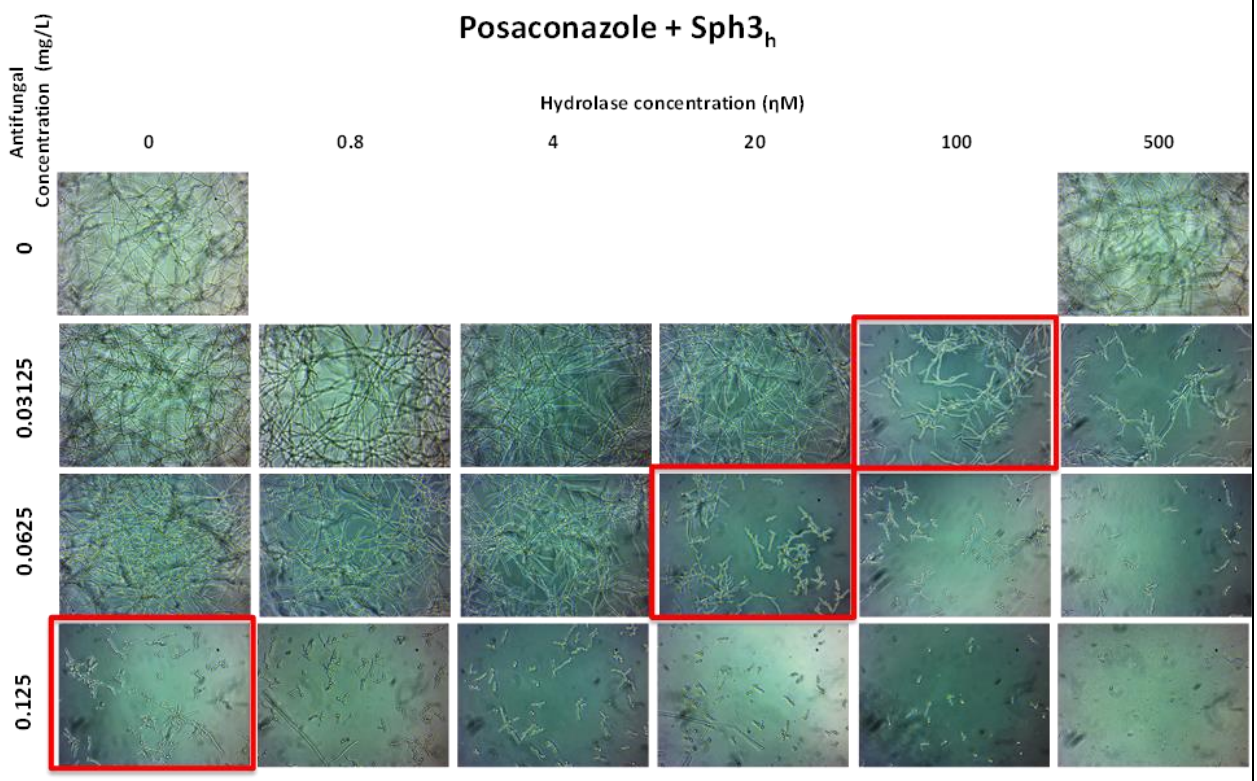


Figure 3. Example of visual scoring. Red boxes indicate the MIC_{90}. The changes in MIC_{90} highlight the significantly increased antifungal activity of posaconazole in the presence of $Sph3_h$.

Results: MIC assay by XTT is a widely used method for measuring cell metabolic activity. However, in direct comparisons of the XTT metabolic assay with visual/fungal biomass growth studies, we observed that GH treatment resulted in artificially high metabolic readings in viable fungi. This observation likely reflects enhanced reagent penetration into exopolysaccharide-deficient hyphae following GH treatment (see section "4. IMPACT" and "5. CHANGES /PROBLEMS"). We therefore elected to replace the XTT metabolic assay with visual scoring of the inhibitory effect of drugs, in absence or presence of GH.

1- Results with a fixed concentration of GH: Initial studies demonstrated that 0.5 µM of all 4 GHs, potentiates the inhibition of fungal growth by drugs of the 3 licensed classes of antifungals: polyenes (amphotericin B), azoles (posaconazole) and echinocandins (caspofungin) (Figure 4).

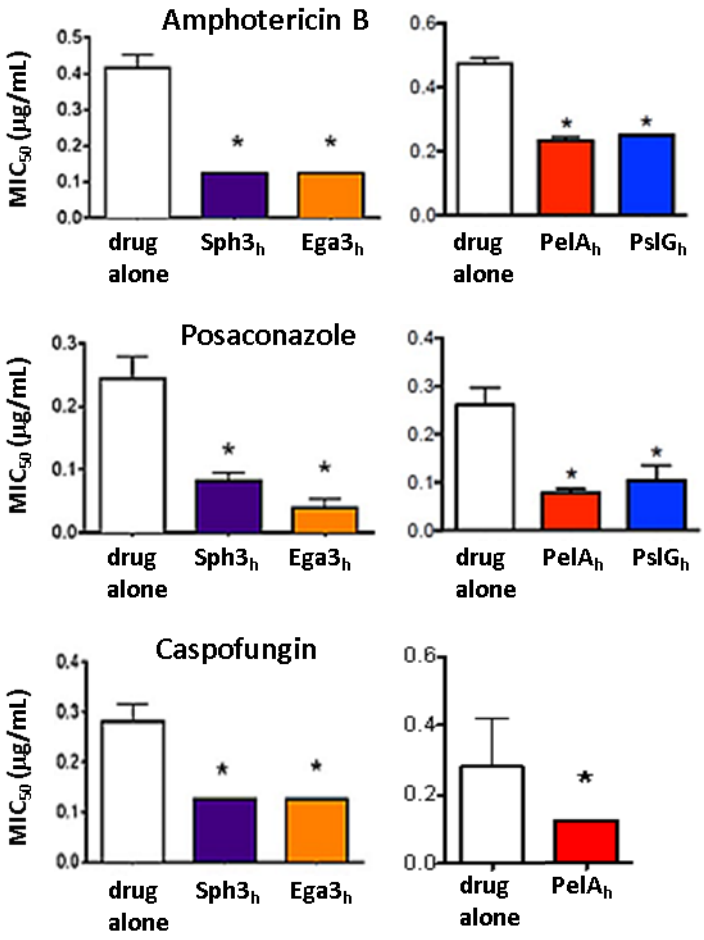


Figure 4: Impact of addition of 0.5 µM of GHs on the MIC_{90} of antifungal drugs. The combination of caspofungin/$PslG_h$ was not included in these initial screening assays but has been evaluated in the full checkerboard assays (Figure 5). * indicates a statistically significant difference compared to drug alone ($p<0.05$).

2- GH-antifungal checkerboard assays

All GH/antifungal combinations demonstrated efficacy against *A. fumigatus* with the exception of the Amphotericin B/PelA combination (Figure 5). A GH concentration of 100 nM appears to be the minimum effective concentration to reduce MIC values for the majority of combinations. The most effective (based on overall MIC value reduction) GH/antifungal combinations tested were posaconazole/$Sph3_h$ and caspofungin/$PelA_h$, and these combinations will be therefore be prioritized for further studies *in vivo*.

Posaconazole-Sph3		
		MEC
Hydrolase conc. (nM)	0	0.500
	0.8	0.333
	4	0.333
	20	0.250
	100	0.208
	500	0.167

Amphotericin B-Sph3		
		MEC
Hydrolase conc. (nM)	0	0.833
	0.8	0.833
	4	0.667
	20	0.500
	100	0.500
	500	0.417

Caspofungin-Sph3		
		MEC
Hydrolase conc. (nM)	0	0.500
	0.8	0.333
	4	0.333
	20	0.250
	100	0.167
	500	0.125

Posaconzole-Ega3		
		MEC
Hydrolase conc. (nM)	0	0.375
	0.8	0.375
	4	0.375
	20	0.3125
	100	0.1875
	500	0.1875

Amphotericin B-Ega3		
		MEC
Hydrolase conc. (nM)	0	0.667
	0.8	0.667
	4	0.667
	20	0.500
	100	0.500
	500	0.500

Caspofungin-Ega3		
		MEC
Hydrolase conc. (nM)	0	0.583
	0.8	0.500
	4	0.333
	20	0.250
	100	0.208
	500	0.208

Posaconazole-PelA		
		MEC
Hydrolase conc. (nM)	0	0.500
	0.8	0.500
	4	0.500
	20	0.500
	100	0.417
	500	0.333

Amphotericin B-PelA		
		MEC
Hydrolase conc. (nM)	0	0.667
	0.8	0.667
	4	0.667
	20	0.667
	100	0.667
	500	0.667

Caspofungin-PelA		
		MEC
Hydrolase conc. (nM)	0	0.833
	0.8	0.417
	4	0.333
	20	0.250
	100	0.208
	500	0.208

Figure 5. Minimal effective concentration (MEC) in μg/mL for posaconazole, amphotericin B and caspofungin as per visual scoring in a fungal inhibition assay in presence of GHs.

Subtask 3: Test $PslG_h/PelA_h$ and $PslG_h/Ega3_h$ in checkerboard combinations with antibiotics against *P. aeruginosa* biofilms. Dr Howell's lab. SOW Time Period: Months 1-9. Completion level = 50 %.

Background: Our preliminary studies indicated that GH treatment enhanced the antimicrobial activity of colistin. We sought to expand these studies to examine the effects of GH therapy on the activity of other antibiotics in checkerboard assays.

Accomplishments:

Choice of an experimental model:
During Year 1 Quarter 1-2 our lab had acquired the material and a vast general knowledge of the Calgary Biofilm Device (CBD), a method we aimed at using to fulfill this part of our work.

After preliminary assays, as explained in detail in the section "5. PROBLEMS /CHANGES", we concluded that this device is not fit for our experimental needs, and we developed an alternate protocol based on our standing biofilm tube assay. In this assay, bacteria are grown for 24 h and then subjected to GH + antibiotic combinations; following incubation, the biofilm is mechanically disrupted and the cells are enumerated using serial dilutions.

During Year 1 Quarter 3, we experienced some difficulties with reproducibility of the drug potentiation assay using our standing biofilm tube assay and therefore while continuing to develop a reliable assay, we also re-visited with the help of our collaborator Dr Parsek (University of Washington) optimizing the protocol for the Calgary Biofilm Device (CBD).

Results:
Using a 1 μM fixed concentration of $PelA_h/PslG_h$ on biofilms of *P. aeruginosa* PA01, that were grown for 24 h at 37°C, we were able to demonstrate significant potentiation of the antibiotics, tobramycin (an aminoglycoside), ciprofloxacin (a fluoroquinolone) and polymyxin. There was a positive effect for neomycin (another aminoglycoside) but the effect was not statistically significant (Figure 6).

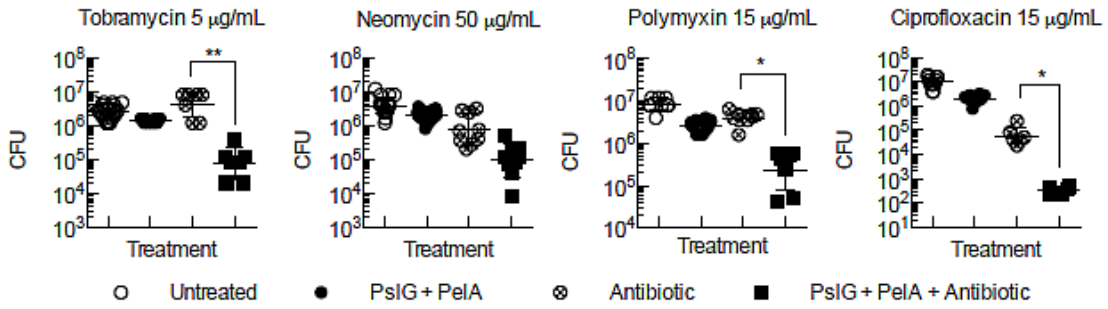


Figure 6. Potentiation of antibiotics efficiency against *P. aeruginosa* by addition of a mix of GHs $PelA_h/PslG_h$ at a 1 μM concentration each. Several concentrations of antibiotics were used and the herein presented results represent the concentrations would gave the highest potentiation. * indicates a significant decrease in CFU counting, with $p \leq 0.05$ by ANOVA test.

Subtask 4: Test candidate hydrolase-antimicrobial combinations in an *in vitro* fluid biofilm culture model system. Dr Howell and Sheppard's labs. SOW Time Period: Months 1-6. Completion level = 25%

Accomplishments:

Methodology: We have successfully developed a method to grow AF under flow biofilm conditions. Briefly, $1.5x10^5$ conidia of RFP-expressing *A. fumigatus* strain Af293 were grown in Brian medium for 9 h. Young hyphae were scraped off, transferred to flow chambers, and incubated at 37°C for 2 h to allow the hyphae to adhere to the chamber surface. Biofilms were then allowed to develop for 24 h at 37°C under constant flow of 1% Brian medium. The resulting biofilms were then stained with FITC-conjugated soy bean agglutinin for GAG detection and imaged by confocal microscopy. Susceptibility of biofilms to GH therapy was evaluated by treating flow-grown biofilms with a 2 μM of the GH of interest for 2 h prior to imaging.

Results: Hyphae stained strongly with SBA-FITC (green), suggesting that GAG is produced under flow conditions (Figure 7A). Consistent with the anti-GAG activity of PslG and Sph3h, GH-treated biofilms exhibited reduced SBA staining compared to the untreated sample. (Figures 7B, 7C)

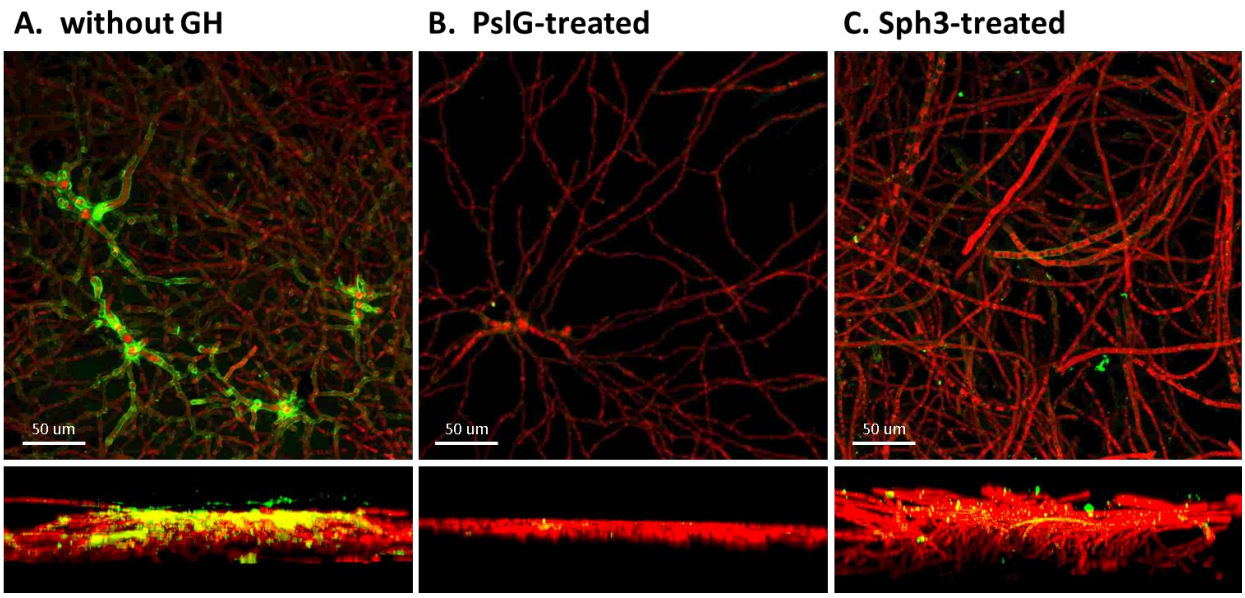


Figure 7**.** SBA-FITC staining of AF biofilms grown under flow conditions. A. Untreated AF biofilms. B. AF biofilms treated with 2 μM of $PslG_h$. C. AF biofilms treated with 2 μM of $Sph3_h$. Vertical Z-stacks were acquired to generate both top-down (top images) and side views (bottom images) for each sample.

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

Subtask 1: Submit documents for Animal use approvals. Dr Sheppard's lab. SOW Time Period: Months 1-6. Completion level = 100%

Accomplishments: IACUC protocol number #2016-7808, was approved by the USAMRMC Animal Care and Use Review Office (ACURO) on 18 Nov 2016. This protocol was previously approved by the McGill University IACUC on 01-JUN-2016.

Subtask 2: Express and purify recombinant $PelA_h$ and $PslG_h$ for subtasks 3 - 4 Dr Howell's lab. SOW Time Period: Months 6-12. Completion level = 100%.

As stated in Major Task 1 – Subtask 1, GH production in our lab is now routine and we can produce sufficient protein, both in quantity and quality (endotoxin-free enzyme, verified activity) to meet all requirements for the other subtasks of Major Task 2.

Subtask 3: Test toxicity of pulmonary administration of GH combinations ($PslG_h$/$PelA_h$ and $PslG_h$/$Ega3_h$ combinations) in immunocompetent mice. Dr Sheppard's lab. SOW Time Period: Months 6-12. Completion level = 100%.

Background:

Preliminary studies in our labs indicated that administration of two doses of 0.75 μg $Sph3_h$ is well tolerated by immunocompromised mice. Our aim is to expand these studies to test of tolerability to all GHs, at dosage as high as 500 μg, administered as single or multiple doses, and in immunocompetent or immunocompromised mice. These studies are critical to guide dose selection for *in vivo* efficacy studies. Subtask 3 focuses on immunocompetent mice and subtask 4 uses immunocompromised animals.

Accomplishments:

Methodology. Recombinant GH enzymes alone and in combination were administered intratracheally at doses ranging from 1-500 μg. Mice were monitored for 7 days, then sacrificed to investigate the sign of pulmonary injury or inflammation. Pulmonary leukocyte recruitment was assessed by flow-cytometry performed on tissue homogenates and by histopathology.

Results:

1- <u>GH monotherapy</u> ($PslG_h$, $PelA_h$, $Sph3_h$, $Ega3_h$):

- GH treatment had no effect on mouse body weight and body temperature. (Figure 8, 9)

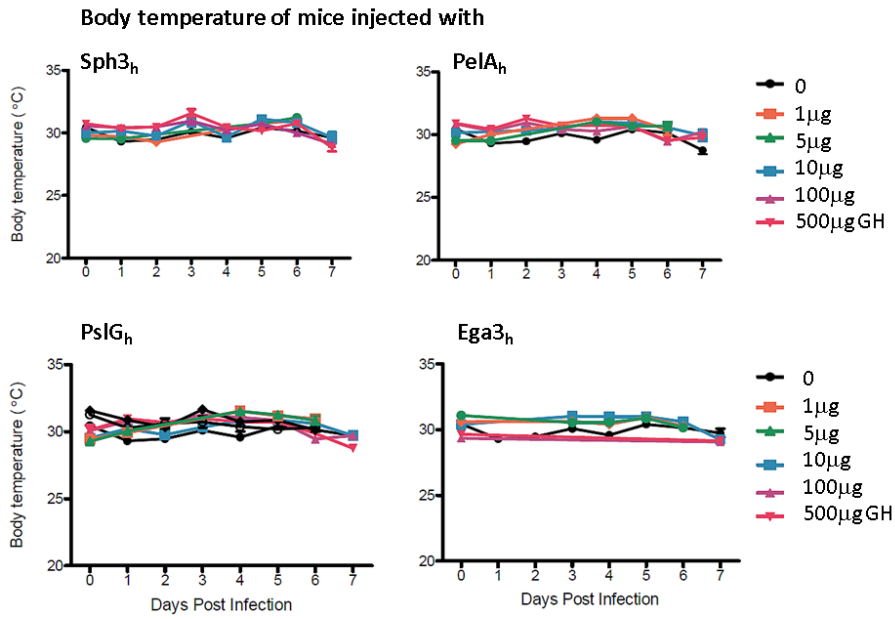


Figure 8. Monitoring of mouse temperature over 7 days after intratracheal injection of the indicated GH in PBS.

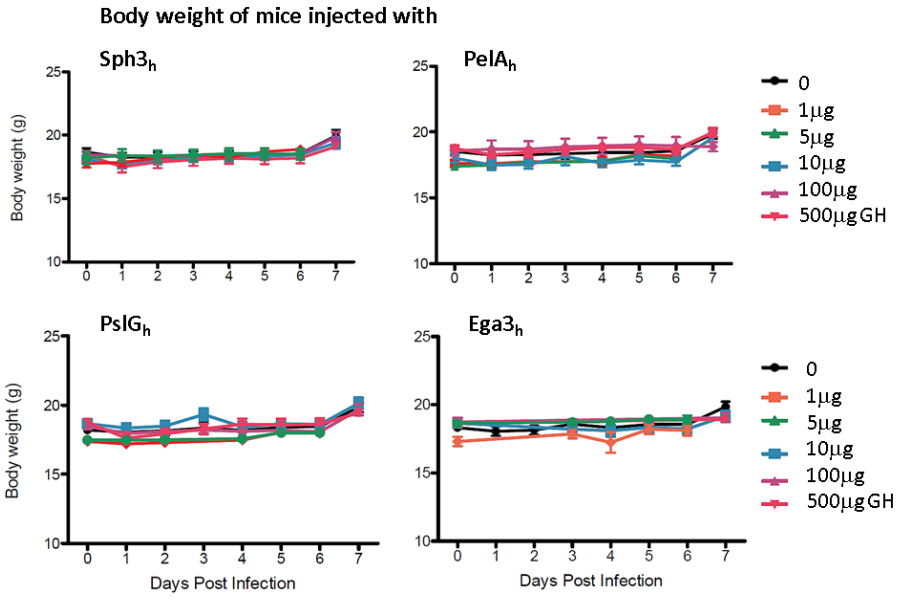


Figure 9. Monitoring of mouse weight over 7 days after intratracheal injection of the indicated GH in PBS.

- Pulmonary leukocyte recruitment: GH therapy was associated with minor increases in pulmonary macrophage numbers (Figure 10), particularly at high doses, suggesting that 500 µg may be the maximal unit dose deliverable to mice. Early experiments with Ega3 suggested a significant increase in pulmonary leukocyte recruitment at doses up to 100 µg. Although this observation could suggest that Ega3 may be less well tolerated than other GHs, these results

were obtained with a single preparation of this enzyme and repeat testing at a higher dose of 500 μg failed to reproduce this response. Repeat studies of lower dose Ega3 are ongoing to confirm the reproducibility of these observations.

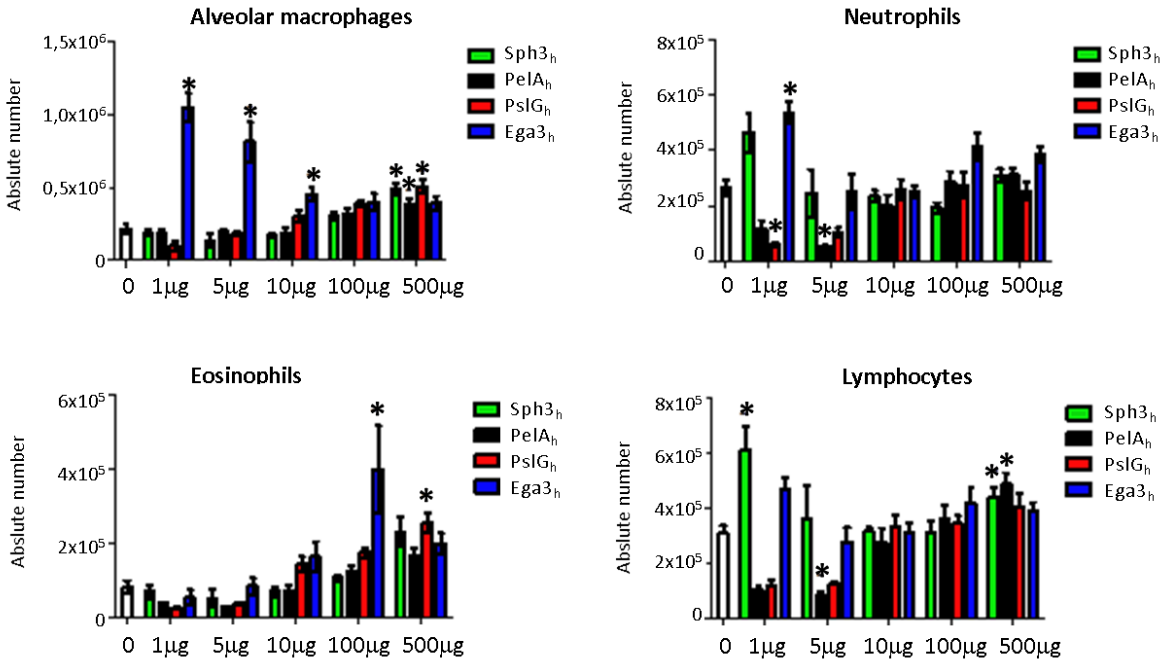


Figure 10. Absolute quantification of leukocyte populations in mouse lungs as measured by flow-cytometry performed on lung homogenates. Lungs were harvested 7 days following treatment with a single dose of the indicated GH. * indicates a significant difference with the untreated group with $p \leq 0.05$ in ANOVA test.

- No differences between treated and untreated mice were detected by histological examination of pulmonary tissue sections (data not shown).
- Pulmonary injury: No differences in lactate dehydrogenase (LDH) release in the BAL fluid were observed between treated and untreated mice suggesting that GH therapy does not induce significant pulmonary injury (Figure 11).

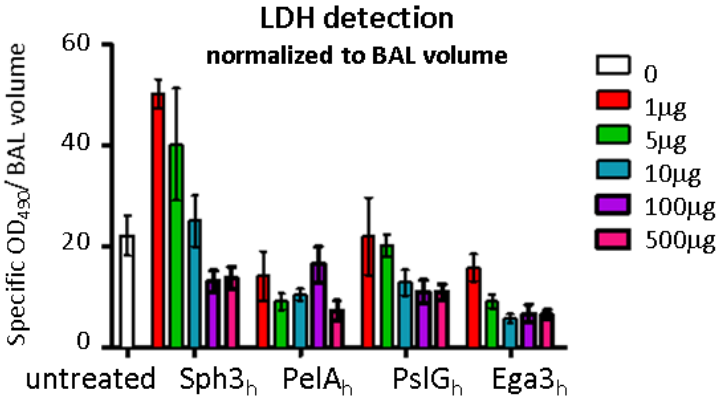


Figure 11. Quantification of lactate dehydrogenase (LDH) activity in the bronchoalveolar fluid harvested from mice 7 days following treatment with a single dose of the indicated GH.

2- <u>Therapy with GH combinations</u> ($PslG_h/PelA_h$ and $PslG_h/Ega3_h$): We have now completed two replicates of these experiments and the data are presented below.

- GH treatment had no effect on mouse body weight and body temperature. (Figure 12, 13)

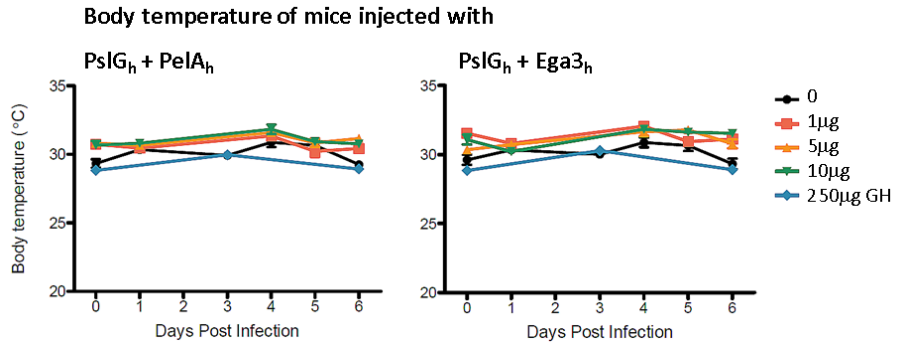


Figure 12. Monitoring of mouse temperature over 7 days after intratracheal injection of the indicated GH combinations in PBS.

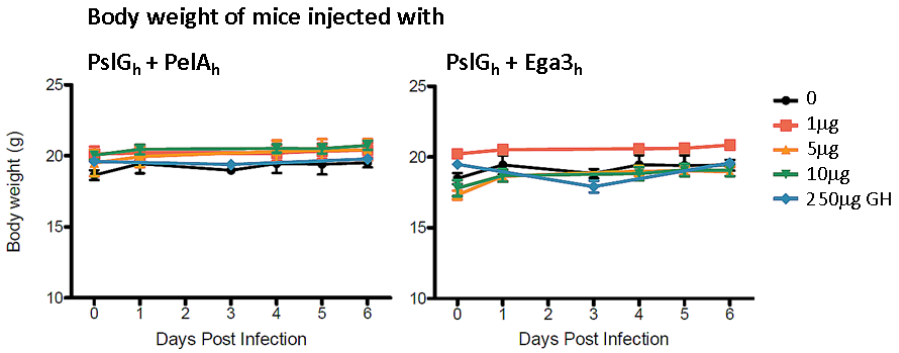


Figure 13. Monitoring of mouse weight over 7 days after intratracheal injection of of the indicated GH combinations in PBS.

- Pulmonary leukocyte recruitment: As with GH monotherapy a significant increase of alveolar macrophages was observed with both GH combinations at the low doses (Figure 14), although interestingly this increase was not observed at the higher combined dose of 250 µg of each GH. In contrast, this higher GH dose was the only one associated with increase in lymphocyte recruitment. No significant variation in recruitment of neutrophils and eosinophil was observed at any dose of GH combination. Collectively these data suggest that effects of GH therapy on pulmonary leukocyte population is not dose-dependent.

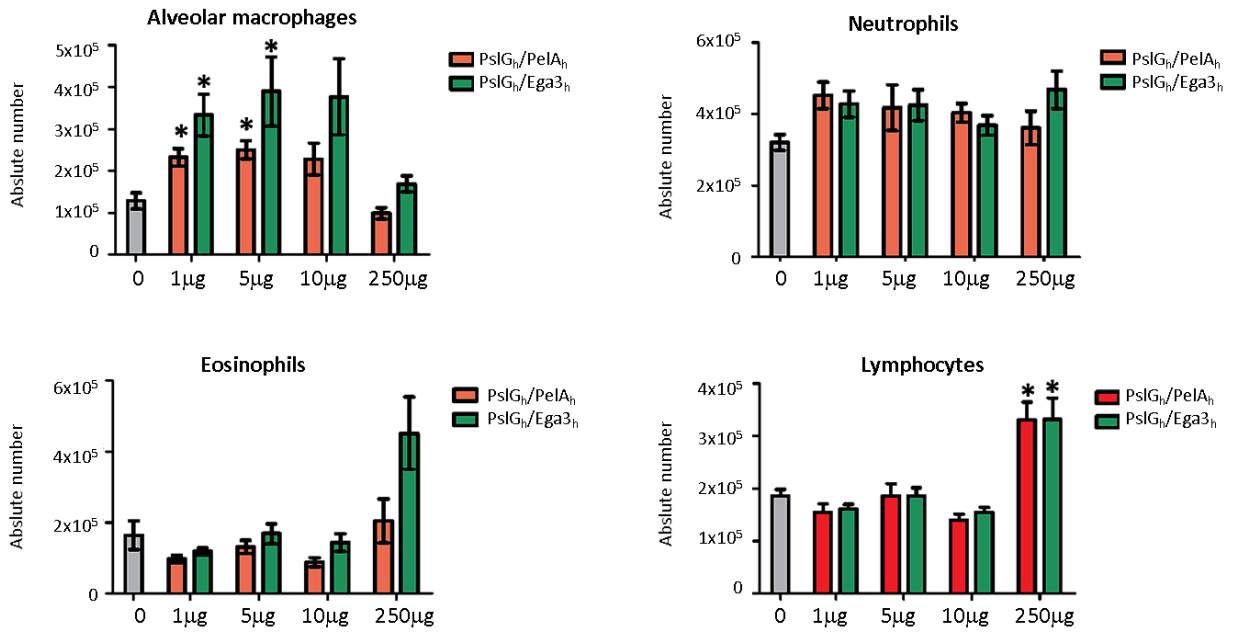


Figure 14. Absolute quantification of leukocyte populations in mouse lungs as measured by flow-cytometry performed on lung homogenates. Lungs were harvested 7 days following treatment with a single dose of the indicated GH combination.

- No differences between treated and untreated mice were detected by histological examination of pulmonary tissue sections (data not shown).
- No differences in lactate dehydrogenase (LDH) release in the BAL fluid were observed between treated and untreated mice (Figure 15).

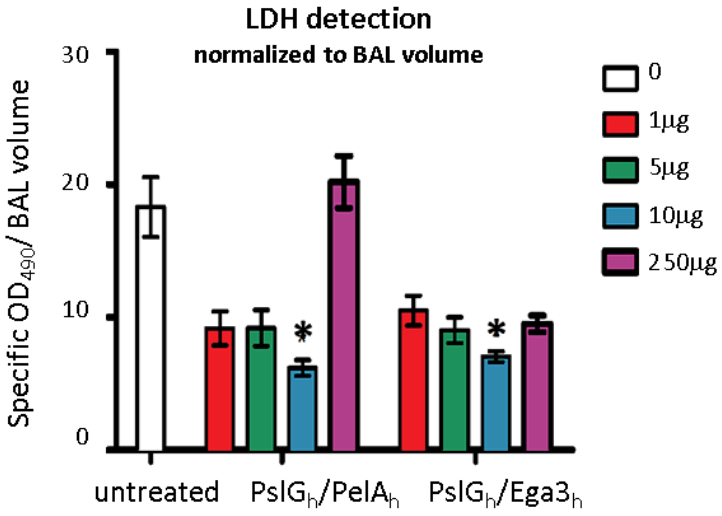


Figure 15. Quantification of lactate dehydrogenase (LDH) activity in bronchoalveolar fluid harvested from mice 7 days following treatment with a single dose of the indicated GH combination.

Subtask 4: Test tolerability of pulmonary administration of hydrolase combinations ($PslG_h$/ $PelA_h$ and $PslG_h$/$Ega3_h$) in immunocompromised mice. Dr Sheppard's lab. SOW Time Period: Months 6-12. Completion level = 50%.

Accomplishments: We have completed the first experiment evaluating the tolerability of $PslG_h$/ $PelA_h$ and $PslG_h$/$Ega3_h$ (250 μg of each GH) in neutropenic mice.

- GH treatment had no effect on mouse body weight and body temperature
- No significant effects on pulmonary leukocytes populations was observed (Figure 16)

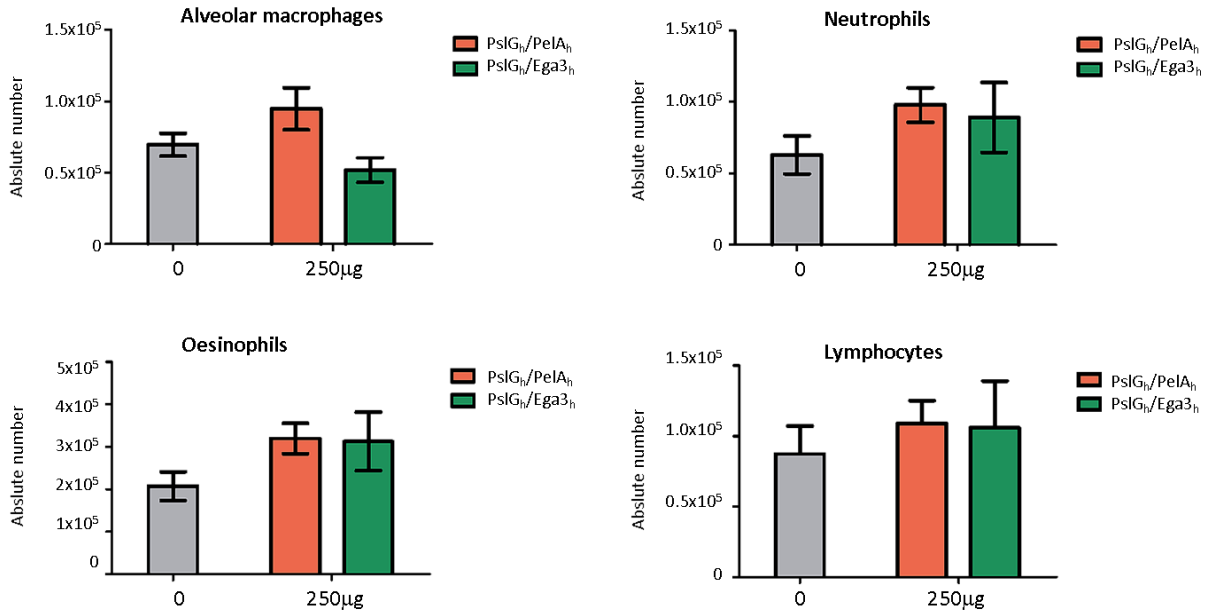


Figure 16. Absolute quantification of leukocyte populations in mouse lungs as measured by flow-cytometry performed on lung homogenates. Lungs were harvested 7 days following treatment with a single dose of the indicated GH combination.

- No differences between treated and untreated mice were detected by histological examination of pulmonary tissue sections (data not shown).
- No differences in lactate dehydrogenase (LDH) release in the BAL fluid were observed between treated and untreated mice (Figure 17).

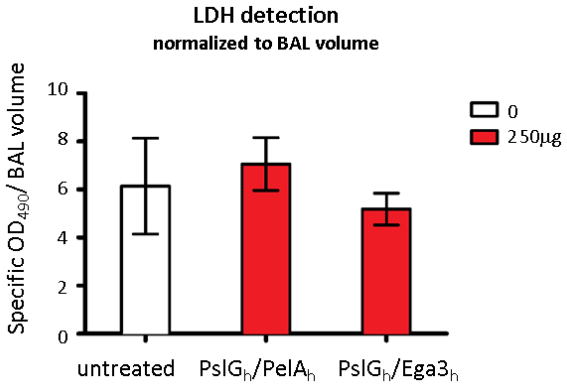


Figure 17. Quantification of lactate dehydrogenase (LDH) activity in the bronchoalveolar fluid harvested from mice 7 days following treatment with a single dose of the indicated GH combination.

MAJOR TASK 3: Pharmacokinetic studies of candidate hydrolases

Subtask 1: Express and purify $Sph3_h$, $Ega3_h$, $PelA_h$ and $PslG_h$ for subtasks 2 - 5. Dr Sheppard's lab. SOW Time Period: Months 6-12. Completion level = 100%.

As stated in Major Task 1 – Subtask 1, GH production in our lab is now routine and we can produce sufficient protein, both in quantity and quality (endotoxin-free enzyme, verified activity) to meet all requirements for the other subtasks of Major Task 3. The necessity to change $Ega3_h$ production from *E. coli* to *P. pastoris* resulted in a delay in preparation of an anti-Ega3 antibody and the completion of subtasks 2, 4 and 5. Anti-Ega3 antibodies are now available and these studies are currently ongoing.

Subtask 2: Test pharmacokinetics of GHs ($Sph3_h$, $Ega3_h$, $PelA_h$ and $PslG_h$/$PelA_h$ and $PslG_h$/$Ega3_h$ 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. SOW Time Period: Months 6-12. Completion level = 50%.

Background:

Determining the pulmonary GH pharmacokinetics is critical for the design of efficacy studies. Polyclonal antibodies that recognize $Sph3_h$, $PelA_h$ or $PslG_h$ have been previously produced by our group, however an anti-$Ega3_h$ antibody had not yet been generated. In this reporting period, we therefore produced and characterized a polyclonal anti-$Ega3_h$ in addition to performing animal experiments to characterize of the pulmonary half-life of recombinant GHs in mice.

Accomplishments:

1- Production and validation of an anti-$Ega3_h$ antibody.

Methodology:
Purified Ega3 was used to generate antiserum from rabbits. The polyclonal antibodies were produced by CedarLane Laboratories (Burlington, Ontario). The production of polyclonal anti-$Ega3_h$ antibodies was evaluated by Western blot using recombinant $Ega3_h$ with and without endoglycosylase (EndoH) treatment.

Results:

Serum from Ega3$_h$-vaccinated rabbits recognizes both glycosylated and deglycosylated Ega3$_h$ (Ega3$_h$, Ega3$_h$ +EndoH) *in vitro* (Figure 18). As indicated the antibody recognizes both forms of the protein.

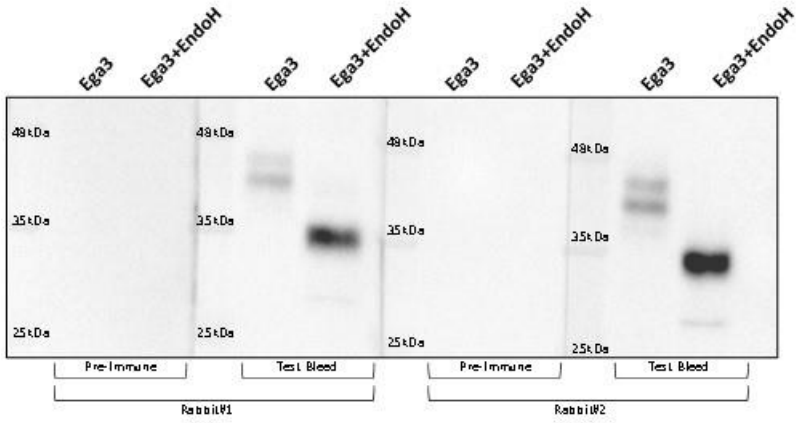


Figure 18. Western-blot of endoglysolase-H treated or untreated purified Ega3$_h$, using rabbit anti-Ega3$_h$ antibody from the first bleed as a primary antibody, and goat-anti-rabbit-HRP antibody as a secondary. GH load was 1 μg per well and the antibody was diluted 1/1000

The terminal bleed confirmed the results observed with the first bleed (Figure 19). The anti-Ega3$_h$ antibody was thereafter tested against Ega3$_h$ as diluted in lung homogenate after tracheal injection in mice.

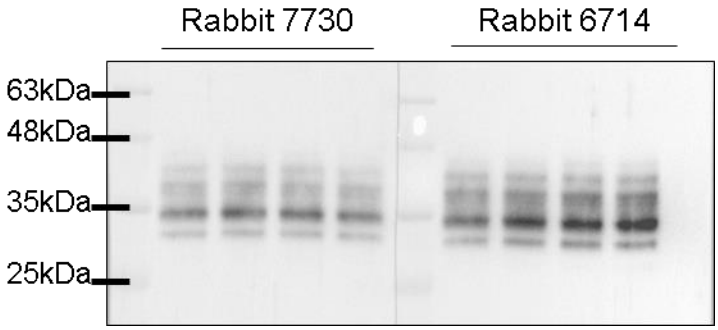


Figure 19. Western-blot of purified Ega3$_h$, using rabbit anti-Ega3$_h$ antibody from terminal bleed as a primary antibody, and goat-anti-rabbit-HRP antibody as a secondary. GH load was 1 μg per well and the antibody was diluted 1/1000.

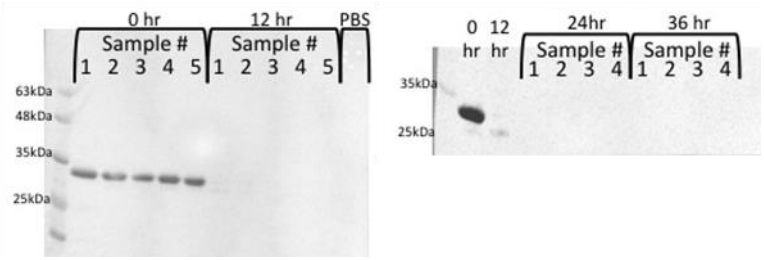
2- <u>Validation of antibody specificity for detection of recombinant GHs in pulmonary tissue</u>

Methodology: Mice were treated intratracheally with 500 μg of each GH. The mice were sacrificed and their lungs harvested at the indicated time points. Homogenized lungs samples were analyzed by western blot analysis to quantify GH concentrations using rabbit anti-GH antibodies. A goat-anti-rabbit-HRP antibody was used as the secondary antibody.

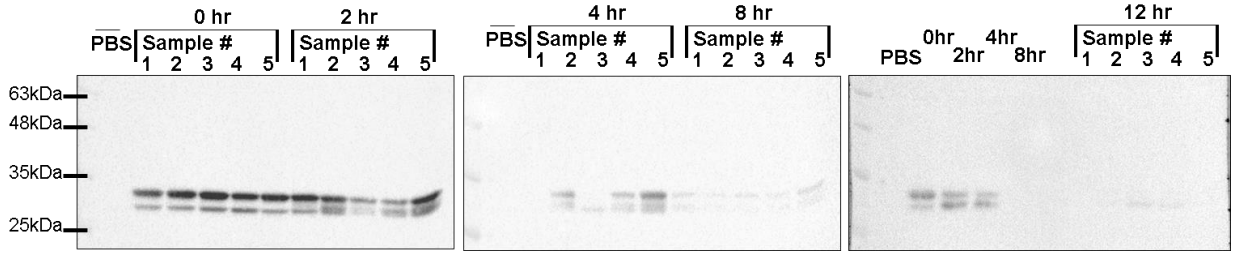
Results:

Western blot analysis showed that anti-GH antibodies detect recombinant GHs in pulmonary tissue (Figure 20).

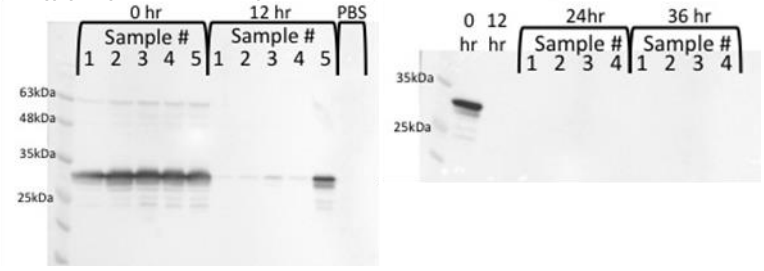
$PelA_h$ (replicate #1)



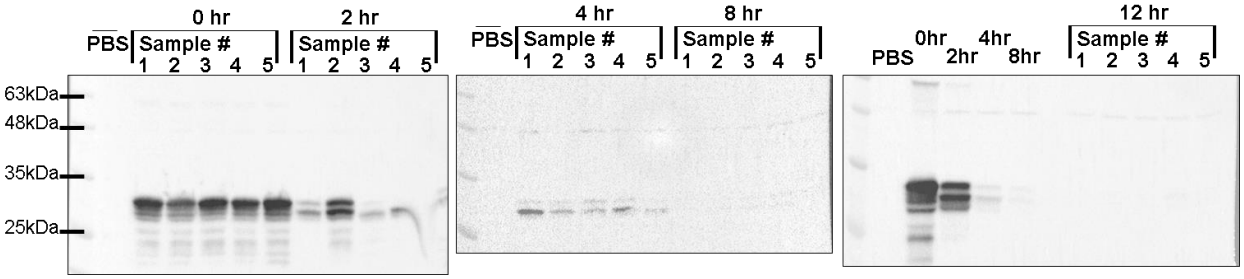
$PelA_h$ (replicate #2)



$Sph3_h$ (replicate #1)



$Sph3_h$ (replicate #2)



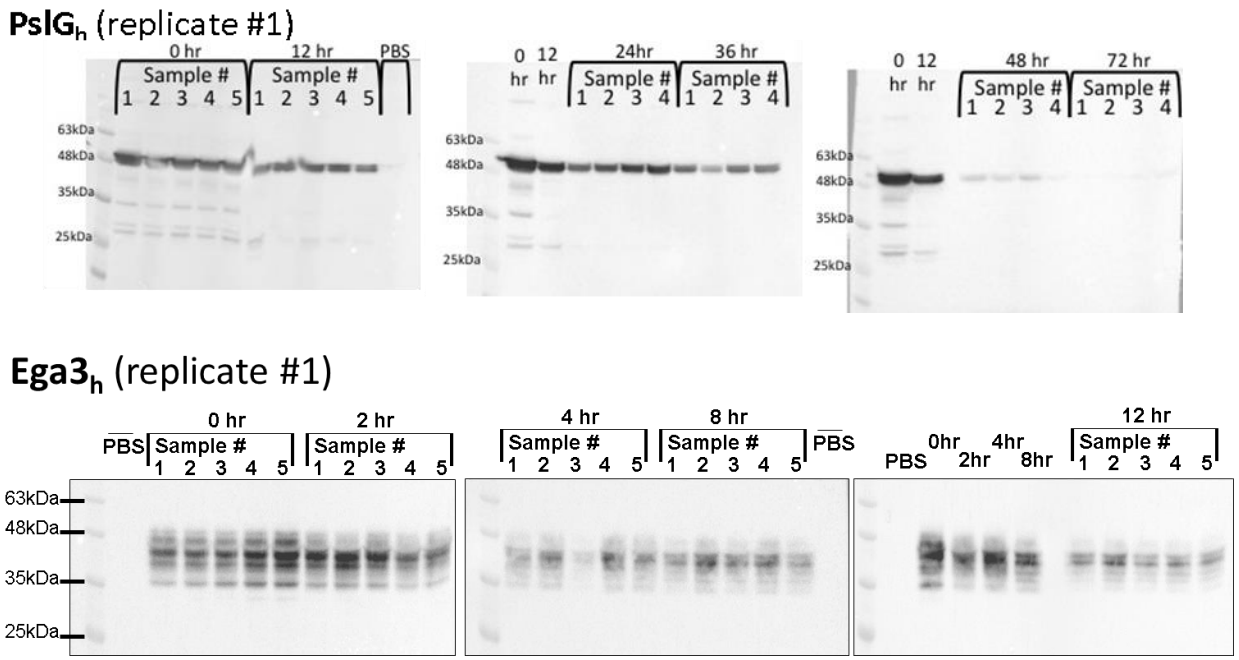


Figure 20. Western-blot of lung homogenates from lung harvested at 0, 12, 24, 36, 48 or 72h after intratracheal treatment with the indicated GH.

3- Pharmacokinetics of recombinant GHs (**Subtask 4**).

Results: Following a single 500 μg intratracheal dose, all GHs were detected in the lung immediately after therapy. Marked variation in the half-lives of individual GH proteins were observed, ranging from 3 to 30 hours (see Table 3)

Hydrolase	Sph3$_h$	PelA$_h$	Ega3$_h$	PslG$_h$
Estimated half-life	3h	4h	8h	30h

Table 3. Estimation of half-life of GHs in mouse lung after intratracheal injection of 500 μg of pure GH in PBS.

Subtask 3: Test pharmacokinetics of hydrolases (Sph3$_h$, Ega3$_h$, PelA$_h$ and PslG$_h$/PelA$_h$ and PslG$_h$/Ega3$_h$ combinations) in immunocompromised 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. Dr Howell and Sheppard's labs SOW Time Period: Months 6-12. Completion level = 0%.

These studies will now be completed in months 13-19.

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 = 50%.

Results from this sub-task are presented in Figure 20 and Table 1.

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

Rachel Corsini and James Stewart were trained by Melanie Lehoux in regard of animal care. They therefore 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.

Brian Hicks, an undergraduate research assistant, was trained by Ira Lacdao in modern biochemical techniques, specifically protein expression and purification.

Natalie Bamford, Perrin Baker, Brendan Snarr presented their results in relation to this grant at several conferences, thus improving their presentation skills. Conferences were: Trends in Medical Mycology Congress. Belgrade, Serbia. October 2017; Canadian Glycobiology Network Symposium, Banff, Canada, May 2017; RI-MUHC IDIGH Research Day 2017, Montreal, Quebec, April 2017.

How were the results disseminated to communities of interest?

Results of our GH studies published in the Proceedings of the National Academy of Sciences (USA) were the subject of a numerous media interviews and reports including: CTV National and local television news as well as TéléQuébec: Les Electrons Libres; newspaper articles in Le Devoir and LaPresse; radio interviews on CBC and CJAD radio; and internet feature articles on CBC and RCI.net.

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

As per SOW, we will complete the unfinished subtasks on which work has been accomplished as described above. In parallel, we will perform the work described in subtasks that are related to the testing of GHs in animal models of acute disease (Major task 5), as well as chronic disease (Major Task 6). Briefly:

Major Task 1 – Subtask 1:
Protein production will continue to support other specific aims.

Major Task 1 – Subtask 2:
Complete.

Major Task 1 – Subtask 3:
Now that we have optimized the assay to show antibiotic potentiation against bacterial biofilm, we planned on performing 2-dimensional checkerboard assays using varying concentrations of drug and GH, in order to determine if potentiation is reproducible and which antibiotic shows the most promising potentiated antibacterial activity. The unexpected departure of PDF Perrin Baker

in early June 2017 and the delay in recruiting his replacement has delayed in the completion of this task.

Major Task 1 – Subtask 4:
We will complete work in this aim by testing GH combinations and confirming the activity of posaconazole/$Sph3_h$ and caspofungin/$PelA_h$ against dynamically grown biofilms

Major Task 2 – Subtask 1:
Complete.

Major Task 2 – Subtask 2:
Complete.

Major Task 2 – Subtask 3:
Complete.

Major Task 2 – Subtask 4:
We will complete our duplicate testing of the tolerability of GHs in immunocompromised mice.

Major Task 3 – Subtask 1:
Complete

Major Task 3 – Subtask 2:
1) The pharmacokinetics of combination GH therapy ($PslG_h$/$PelA_h$ and $PslG_h$/$Ega3_h$) will be tested in immunocompetent mice
2) Using western blot analysis as a gold standard we have begun to develop an ELISA for future pharmacokinetic analyses. To date we have established the ELISA protocol for $PelA_h$ and $PslG_h$ using purified protein, and are currently developing the ELISA assay for Sph3 and Ega3. These assays will be used in the preclinical development of the enzymes.

Major Task 3 – Subtask 3:
The assays outlined in Subtask 2 will be repeated in immunocompromised mice.

Major Task 4 –
Once we have defined the half-life of each GH we will evaluate the need for Major task 4 (modification of hydrolases).

Major Task 5: Test GHs for activity in animal models of acute disease
Has been initiated for completion by month 30.

Major Task 6: Test GHs for activity in animal models of chronic disease
To be initiated for completion by month 30.

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

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

Our demonstration that microbial GHs can disrupt biofilms has generated significant interest in the scientific community. A recent review in Nature Microbiology Reviews highlighted the potential of GH therapy and we have been invited to submit an editorial describing the potential of these therapeutics in the fight against antimicrobial resistant organisms.

What was the impact on other disciplines?

XTT metabolic assay
This assay is a commonly used method to study fungal biofilms and the activity of antimicrobial agents against biofilm-grown fungi. Our finding that loss of exopolysaccharide matrix can lead to artificially high metabolic readings suggests an important limitation to this assay that was previously unknown.

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:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

No significant changes beyond the minor modifications to experimental techniques detailed below.

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

Major Task 1 –Subtask 2:
XTT metabolic assay is the method we originally planned to use for measuring antifungal drug potentiation by GHs. This assay is a colorimetric assay that relies on intracellular penetration of the XTT reagent into the fungi where it is metabolized. Due to the loss of hyphal exopolysaccharide due to GH treatment, uptake of the XTT reagent by fungi is enhanced, leading to artificially high metabolic readings in the presence of GH enzymes. Therefore, the XTT assay has a limited range of reliability and accuracy. We have therefore elected to use visual scoring of the inhibitory effect of drugs, in absence or presence of GH. Visual scoring is performed as per CLSI (Clinical & Laboratory Standards Institute) guidelines in a blinded fashion.

Major Task 1 – Subtask 3:

1) Progress on this task was delayed due to the unanticipated departure of postdoctoral fellow Perrin Baker (Howell Lab) in early June 2017. After advertising the position and interviewing candidates, Dr. Raju was recruited and joined the Howell Lab on September 12, 2017. Completion of these studies has been a priority for Dr. Raju since her recruitment.

2) Antibiotic Potentiation
Despite expertise using the Calgary Biofilm Device (CBD) we have concluded that this device is not suited for the proposed experiments: the CBD is used to qualitatively determine the amount of antibiotic required to kill cells in a biofilm state; this means that all biofilm biomass must be removed from the 96 polystyrene pegs into a 96-well plate, as the plate is read for bacterial growth. Unfortunately, the biofilms produced in the lab cannot easily and consistently be removed from pegs, thus leading to inconsistent results. From other antibiotic potentiation assays we have also found that optimal antibiotic potentiation occurs at an antibiotic concentration that is sufficiently high to kill planktonic (free-swimming cells) but not biofilm embedded bacterial cells.
To overcome the CBD issues and with the knowledge we have gained from other experiments, we have moved to using a standing biofilm tube assay, previously published in our 2016 Science Advances paper. Our aim was to test antibiotic concentrations which show 1 log or more killing and use these concentrations with a single dose of each enzyme combination that is sufficient to disrupt the biofilm. This methodology was supposed to increase both the feasibility and the reproducibility of the assay.
While we continued to develop the protocol for the drug potentiation assays using our standing biofilm tube assay, we also decided to re-visit and optimize our Calgary Biofilm Device protocol. With the help of our collaborator Dr Parsek (University of Washington), we were able to increase biofilm formation, and results from Dr. Parsek's lab, using fixed concentration of $PelA_h$/$PslG_h$ on biofilms of *P. aeruginosa* PA01 are encouraging that this method is now functional in our hands. We now need to transfer the protocol developed by Dr. Parsek and reproduce these results in house and therefore will continue to explore the use of the CBD and our standard biofilm assay to complete the potentiation experiments outlines.

3) Choice of *P. aeruginosa* strain for *in vivo and in vitro* studies.
We had proposed to test several *P. aeruginosa* strains that produce different biofilm polysaccharide profiles. PA14 strain was selected to represent strains which have been reported to produce Pel. However, work from our group has now demonstrated that although PA14 makes abundant Pel-dependent biofilms at 25°C, biofilm formation is significantly reduced at 37°C likely due to a marked reduction in Pel production (Figure 21). Consequently, while treatment with GHs reduces residual biomass of biofilms grown at either temperature, this reduction is only significant when biofilms are grown at 25°C. We will therefore switch to the use of clinical isolates from patients with chronic lung disease, which have been demonstrated to produce Pel and Psl at 37°C and are therefore more suitable for animal studies. Verification of the ability of these strains to form biofilms at 37°C is in progress.

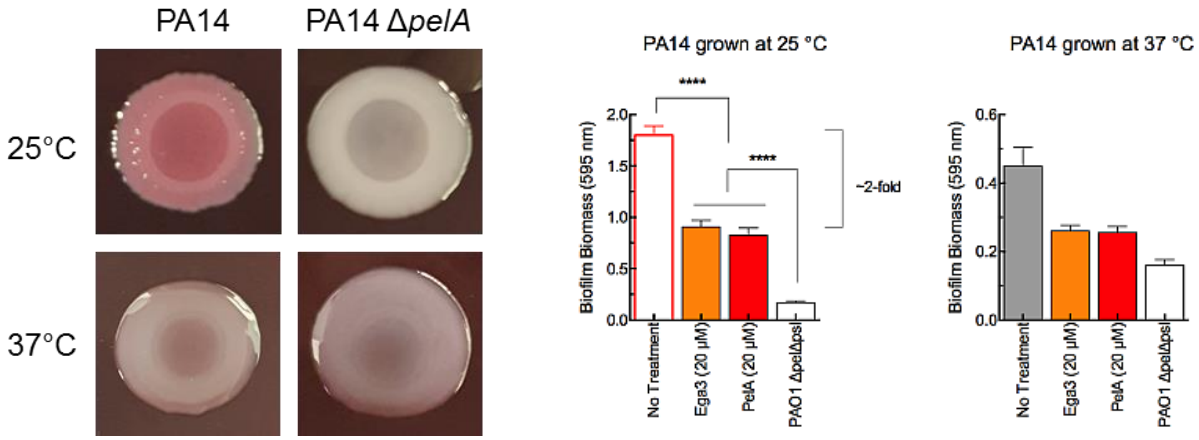


Figure 21: Thermosensitivity of *P. aeruginosa* biofilm production. Left panel: Wrinkly biofilms are only produced in PA14 at 25°C. Right panel: Quantification of the biofilm biomass produced by *P. aeruginosa* at different temperatures and subsequently disrupted by GH treatment. * indicates a significant decrease in biomass, with $p \leq 0.05$ by ANOVA test.

Major Task 3 - Subtasks 2, 3, 4:
As reported above (Problems in Major Task 1 – Subtask 1), production of endotoxin free $Ega3_h$ required shifting to a new production system. This issue has now been resolved, and we are now able to perform both the IV injection in rabbits for the production of specific antibody and the intratracheal inoculation in mice for the pulmonary GH pharmacokinetic experiments. We are on track to finish all these studies within the coming 6 months.

Changes that had a significant impact on expenditures

No change with a significant impact on expenditures to be reported

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.

No significant change in use or care of vertebrate animals to be reported

Significant changes in use of biohazards and/or select agents

No significant change in use of biohazards and/or select agents to be reported

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

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications

Publications

1. Snarr BD, Baker P, Bamford NC, Sato Y, Liu H, Lehoux M, Gravelat FN, Ostapska H, Baistrocchi SR, Cerone RP, Filler EE, Parsek MR, Filler SG, Howell PL, and Sheppard DC. "Microbial glycoside hydrolases as antibiofilm agents with cross-kingdom activity." PNAS, published online June 20, 2017, doi: 10.1073/pnas.1702798114.

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.

Presentations

1. P.L. Howell. *Microbial biofilms: Mechanisms to therapeutics*. SickKids Summer Student Seminar Program, Toronto, Ontario, June 22, 2017
2. P.L. Howell. *Microbial biofilms: Mechanisms to therapeutics*. Ontario Public Health, Toronto, Ontario, May 22, 2017.
3. P. L. Howell. *Microbial biofilms: Mechanisms to therapeutics*. Understanding Biology through Structure, Santa Fe, New Mexico, May 13-17, 2017.
4. P.L. Howell. Glycoside hydrolases as therapeutics. Gordon Research Conference, Cellulases and other carbohydrate active enzymes, New Hampshire, July 24-28, 2017.
5. DC Sheppard. *Glycoside GHs as novel antibiofilm therapeutics*. US Department of Defense, CMDRP, Fort Detrick, Maryland, April 4, 2017.
6. DC Sheppard. *Glycoside GHs as novel antibiofilm therapeutics*. GlycoNET Network Centre of Excellence Annual General Meeting, Banff, Alberta, April 4, 2017.
7. DC Sheppard. Aspergillus *biofilm exopolysaccharide – from virulence factor to therapeutic target*. 29th Fungal Genetics Conference, Asilomar, California, March 19, 2017.
8. Sheppard DC, Invited Speaker, 29th Fungal Genetics Conference. Asilomar, California. "Breaking the mold: From host-pathogen interactions to novel therapeutics for Aspergillus disease." March 17 2017
9. Sheppard DC, Invited Speaker, IUBMB Frontiers in Glycoscience: Host-pathogen interactions. Taipei, Taiwan. *Biofilm exopolysaccharides at the host-pathogen interphase*. Dec 12 2016
10. Sheppard DC, Invited Speaker, Immunocompromised Host Society Meeting, Santiago, Chile. *Enhancing delivery of antifungals to pulmonary lesions – intracellular antifungals and antibiofilm therapeutics*. Nov 14 2016.

Posters

1. Snarr BD, Baker P, Bamford NC, Sato Y, Lui H, Lehoux M, Gravelat FN, Baistrocchi SR, Parsek MR, Filler SG, Howell PL, Sheppard DC. *Microbial glycoside hydrolases as antibiofilm agents with cross-kingdom activity*. Trends in Medical Mycology Congress. Belgrade, Serbia. October 7, 2017
2. Bamford NC., Snarr BD., Gravelat FN., Little DJ., Le Mauff F., Lee MJ., Robinson H., Sheppard DC., Howell PL. *A novel glycoside hydrolase is required for galactosaminogalactan biosynthesis by* Aspergillus fumigatus. Canadian Glycobiology Network Symposium. Banff, Canada. May 11 2017.
3. P. Baker, H. Silver, P.J. Hill, M.J. Pestrak, M. Litvak, M. Post, M.R. Parsek, D.J. Wozniak, P.L. Howell. *Developing enzyme-based bacterial biofilm disruptors by targeting exopolysaccharides*. Canadian Glycomics Symposium 2017, Banff, Alberta, May 10-12, 2017.
4. N.C. Bamford, B.D. Snarr, F.N. Gravelat, D.J. Little, F. Le Mauff, M.J. Lee, H. Robinson, D.C. Sheppard, P.L. Howell. *A novel glycoside hydrolase is required for galactosaminogalactan biosynthesis by* Aspergillus fumigatus. Canadian Glycomics Symposium 2017, Banff, Alberta, May 10-12, 2017.
5. B.D. Snarr, P. Baker, N.C. Bamford, Y. Sato, H. Lui, M. Lehoux, S.R. Baistrocchi, S.G. Filler, P.L. Howell, D.C. Sheppard. *Microbial glycoside hydrolases as antibiofilm agents with cross-*

kingdom activity against both bacteria and fungi. RI-MUHC IDIGH Research Day 2017, Montreal, Quebec, April 21, 2017.

- **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).

- **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	12
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: 7.5
Contribution to Project: Co-responsible for the animal work

Name: *Brendan Snarr*
Project Role: PhD candidate
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 12
Contribution to Project: Responsible for the *in vitro* dynamic fluid biofilm cultures of *A. fumigatus*

Name: *James Stewart*
Project Role: Masters candidate
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 9
Contribution to Project: Co-responsible for the test of the GHs in checkerboard combinations with antifungals against A. fumigatus biofilms

Name: *Hanna Ostapska*
Project Role: PhD candidate
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 3
Contribution to Project: Co-responsible for the test of the GHs in checkerboard combinations with antifungals *against A. fumigatus* biofilms

Name: *Caitlin Zacharias*
Project Role: PhD candidate
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 3
Contribution to Project: Co-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:	3
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

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

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

Grant Log #PR150786

Award #W81XWH-16-1-0283

PI: Donald Sheppard **Org: Research Institute of the McGill University Health Centre** **Award Amount:** $1,134,417.00

Study/Product Aim(s)

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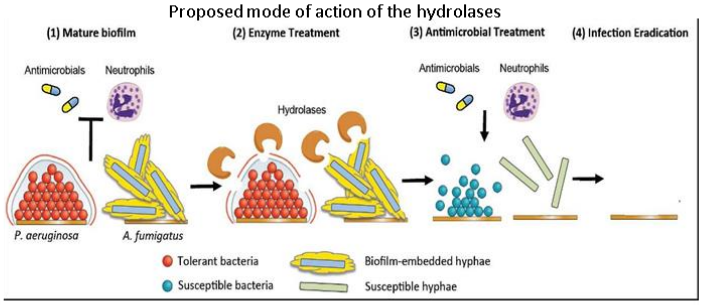
- Aim 3. To evaluate candidate hydrolases alone and in combination with antimicrobial agents in the treatment of experimental *A. fumigatus* and *P. aeruginosa* pulmonary infections in vivo.

Approach

A. fumigatus and *P. aeruginosa* are two lung opportunistic pathogens that embed themselves in a biofilm, becoming therefore more resistant to drugs. We will demonstrate here the possibility to use four enzymes, two hydrolases from fungal origin, two from bacterial origin, to render microorganisms more susceptible to antimicrobials *in vivo*. We also determine the concentration of hydrolases that is both efficient and well tolerated by the host. Our purpose is to generate data that will allow the start of clinical trials.

Proposed mode of action of the hydrolases



Accomplishments in 2016-2017: Purification of all hydrolases. Enhancement of activity of several antifungal drugs on *A. fumigatus* biofilm when combined to the hydrolases in static culture and in dynamic culture. Showed an absence of toxicity of hydrolases in immunocompetent mice. Production of anti-Ega3 antibody , and for all 4 hydrolases, validation of hydrolase detection in lung tissues.

Timeline and Cost

Activities CY	16	17	18	19
Specific Aim 1 - Major task 1				
Specific Aim 2 - Major task 2				
Specific Aim 2 - Major task 3				
Specific Aim 2 - Major task 4				
Specific Aim 3 - Major task 5				
Specific Aim 3 - Major task 6				
Specific Aim 3 - Major task 7				
Estimated Budget ($K)	**$28**	**$386**	**$411**	**$309**

Updated: Oct 14, 2017

Goals/Milestones

CY17 Goals

- ☐ Identification of hydrolase-antimicrobial combination that synergize against *A. fumigatus* and *P. aeruginosa*.
- ☑ Obtain ACURO approval
- ☐ Evaluation of pulmonary toxicity of candidate hydrolase regimens *in vivo*
- ☐ Evaluation of pharmacokinetics of candidate hydrolase regimens

CY18 Goals

- ☐ Development of stable candidates hydrolases (as required)

CY19 Goals

- ☐ Efficacy of candidate hydrolase regimens in the treatment of acute infection with *A. fumigatus* and *P. aeruginosa* confirmed
- ☐ Efficacy of candidate hydrolase regimens in the treatment of chronic infection with *A. fumigatus* and *P. aeruginosa* confirmed
- ☐ Demonstrate proof-of-concept for candidate hydrolases for use in treatment of *A. fumigatus* and *P. aeruginosa*. Ready to initiate trials of delivery systems and detailed pharmacodynamics experiments as a prelude to Phase I clinical trials.

Budget Expenditure to Date:
Projected Expenditure: $ 317,000
Actual Expenditure: $ 219,700

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

Microbial glycoside hydrolases as antibiofilm agents with cross-kingdom activity

Brendan D. Snarr[a,b,1], Perrin Baker[c,1], Natalie C. Bamford[c,d,1], Yukiko Sato[a,b], Hong Liu[e], Mélanie Lehoux[b], Fabrice N. Gravelat[b], Hanna Ostapska[a,b], Shane R. Baistrocchi[a,b], Robert P. Cerone[a,b], Elan E. Filler[e], Matthew R. Parsek[f], Scott G. Filler[e,g], P. Lynne Howell[c,d,2], and Donald C. Sheppard[a,b,2]

[a]Department of Microbiology and Immunology, McGill University, Montreal, QC, H3A 2B4, Canada; [b]Department of Medicine, Infectious Diseases and Immunity in Global Health Program, Centre for Translational Biology, McGill University Health Centre, Montreal, QC, H4A 3J1, Canada; [c]Program in Molecular Medicine, Research Institute, The Hospital for Sick Children, Toronto, ON, M5G 1X8, Canada; [d]Department of Biochemistry, University of Toronto, Toronto, ON, M5S 1A8, Canada; [e]Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA 90502; [f]Department of Microbiology, University of Washington, Seattle, WA 98195; and [g]David Geffen School of Medicine at UCLA, University of California, Los Angeles, CA 90024

Edited by Scott J. Hultgren, Washington University School of Medicine, St. Louis, MO, and approved March 30, 2017 (received for review March 2, 2017)

Galactosaminogalactan and Pel are cationic heteropolysaccharides produced by the opportunistic pathogens *Aspergillus fumigatus* and *Pseudomonas aeruginosa*, respectively. These exopolysaccharides both contain 1,4-linked *N*-acetyl-D-galactosamine and play an important role in biofilm formation by these organisms. Proteins containing glycoside hydrolase domains have recently been identified within the biosynthetic pathway of each exopolysaccharide. Recombinant hydrolase domains from these proteins ($Sph3_h$ from *A. fumigatus* and $PelA_h$ from *P. aeruginosa*) were found to degrade their respective polysaccharides in vitro. We therefore hypothesized that these glycoside hydrolases could exhibit antibiofilm activity and, further, given the chemical similarity between galactosaminogalactan and Pel, that they might display cross-species activity. Treatment of *A. fumigatus* with $Sph3_h$ disrupted *A. fumigatus* biofilms with an EC_{50} of 0.4 nM. $PelA_h$ treatment also disrupted preformed *A. fumigatus* biofilms with EC_{50} values similar to those obtained for $Sph3_h$. In contrast, $Sph3_h$ was unable to disrupt *P. aeruginosa* Pel-based biofilms, despite being able to bind to the exopolysaccharide. Treatment of *A. fumigatus* hyphae with either $Sph3_h$ or $PelA_h$ significantly enhanced the activity of the antifungals posaconazole, amphotericin B, and caspofungin, likely through increasing antifungal penetration of hyphae. Both enzymes were noncytotoxic and protected A549 pulmonary epithelial cells from *A. fumigatus*-induced cell damage for up to 24 h. Intratracheal administration of $Sph3_h$ was well tolerated and reduced pulmonary fungal burden in a neutropenic mouse model of invasive aspergillosis. These findings suggest that glycoside hydrolases can exhibit activity against diverse microorganisms and may be useful as therapeutic agents by degrading biofilms and attenuating virulence.

biofilm | *Aspergillus* | *Pseudomonas* | therapeutics | exopolysaccharide

The mold *Aspergillus fumigatus* and the Gram-negative bacterium *Pseudomonas aeruginosa* are opportunistic pathogens that cause pulmonary infection in immunocompromised patients and individuals who suffer from chronic lung diseases such as cystic fibrosis and bronchiectasis. *A. fumigatus* is the second most common nosocomial fungal infection (1), and ∼10% of all nosocomial bacterial infections are caused by *P. aeruginosa* (2). Mortality associated with *P. aeruginosa* infections is high (3) and has increased with the emergence of multi- and even pan-resistance to antibiotics (3, 4). Similarly, invasive aspergillosis is associated with mortality rates of up to 50% (5), and increasing rates of antifungal resistance have been reported worldwide (6). These factors underscore the urgent need for new effective therapies for these infections.

Although *A. fumigatus* and *P. aeruginosa* are members of different taxonomic kingdoms, both produce biofilms that constitute a protective lifestyle for the organism. Biofilms are complex communities of microorganisms that grow embedded in an extracellular matrix composed of DNA, protein, and exopolysaccharide (7). Biofilm formation provides a significant advantage to these organisms because the matrix mediates adherence to host cells (8, 9) and aids in the resistance to both antimicrobial agents (10, 11) and host–immune defenses (12, 13). *A. fumigatus* biofilm formation depends on the cationic polysaccharide galactosaminogalactan (GAG), a heteroglycan composed of α1,4-linked galactose and *N*-acetyl-D-galactosamine (GalNAc) that is partially deacetylated (14, 15). In comparison, *P. aeruginosa* has the genetic capacity to produce three biofilm exopolysaccharides: alginate, Psl and Pel (16). GAG shares several similarities with Pel, which has been identified as a cationic heteroglycan composed of 1,4-linked GalNAc and *N*-acetyl-D-glucosamine (GlcNAc) (17). Like GAG, the cationic nature of Pel results from partial deacetylation of the polymer (17). Most clinical and environmental isolates of *P. aeruginosa* use Pel and Psl during biofilm formation (18). Alginate is dispensable for biofilm formation and is only observed in chronic pulmonary infection when strains switch to a mucoid phenotype (18, 19).

Strains of *Aspergillus* and *P. aeruginosa* with impaired GAG, or Pel and Psl biosynthesis exhibit attenuated virulence (20, 21), suggesting that targeting these exopolysaccharides may be a

Significance

The production of biofilms is an important strategy used by both bacteria and fungi to colonize surfaces and to enhance resistance to killing by immune cells and antimicrobial agents. We demonstrate that glycoside hydrolases derived from the opportunistic fungus *Aspergillus fumigatus* and Gram-negative bacterium *Pseudomonas aeruginosa* can be exploited to disrupt preformed fungal biofilms and reduce virulence. Additionally, these glycoside hydrolases can be used to potentiate antifungal drugs by increasing their hyphal penetration, to protect human cells from fungal-induced injury, and attenuate virulence of *A. fumigatus* in a mouse model of invasive aspergillosis. The findings of this study identify recombinant microbial glycoside hydrolases as promising therapeutics with the potential for antibiofilm activity against pathogens across different taxonomic kingdoms.

Author contributions: B.D.S., P.B., N.C.B., P.L.H., and D.C.S. designed research; B.D.S., P.B., N.C.B., Y.S., H.L., M.L., F.N.G., H.O., S.R.B., R.P.C., and E.E.F. performed research; M.R.P. contributed new reagents/analytic tools; B.D.S., P.B., N.C.B., Y.S., H.L., M.L., F.N.G., S.R.B., S.G.F., P.L.H., and D.C.S. analyzed data; and B.D.S., P.B., N.C.B., M.R.P., S.G.F., P.L.H., and D.C.S. wrote the paper.

Conflict of interest statement: A patent has been filed describing the utility of the glycoside hydrolases as antibiofilm therapeutics (CA2951152 A1, WO2015184526 A1). B.D.S., P.B., N.C.B., P.L.H., and D.C.S. are listed as inventors.

This article is a PNAS Direct Submission.

[1]B.D.S., P.B., and N.C.B. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: don.sheppard@mcgill.ca or howell@sickkids.ca.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1702798114/-/DCSupplemental.

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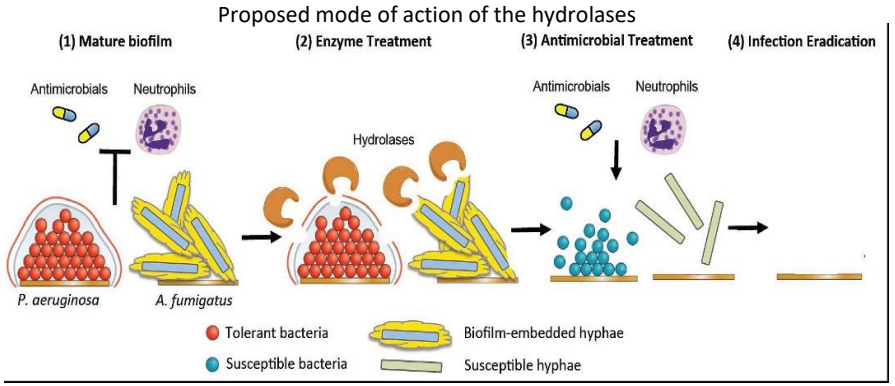

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