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.

useful therapeutic strategy. We previously demonstrated that recombinant glycoside hydrolases $PelA_h$ and $PslG_h$, encoded in the *pel* and *psl* operons of *P. aeruginosa*, respectively, target and selectively hydrolyze the Pel and Psl exopolysaccharide components of the *Pseudomonas* biofilm matrix (22). Treatment with these enzymes rapidly disrupts established biofilms, increasing the susceptibility of *P. aeruginosa* to human neutrophil killing and potentiation of the antibiotic colistin (22).

Our recent work on *Aspergillus* has identified a cluster of five genes that encode the proteins necessary for GAG biosynthesis (15). As with *P. aeruginosa*, we found that the product of one of these genes contains a glycoside hydrolase domain, $Sph3_h$, which is capable of hydrolyzing purified and cell wall-associated GAG (23). In the present study, we assessed the therapeutic potential of $Sph3_h$ in disrupting fungal biofilms. We establish that the exogenous addition of $Sph3_h$ is capable of rapidly disrupting existing biofilms of this organism at nanomolar concentrations. Additionally, we demonstrate cross-kingdom activity, because the *P. aeruginosa* glycoside hydrolase, $PelA_h$, was able to disrupt *A. fumigatus* biofilms. Whereas $Sph3_h$ was able to bind Pel, it was unable to disrupt preformed *P. aeruginosa* Pel-mediated biofilms. Treatment with $Sph3_h$ or $PelA_h$ increased the susceptibility of wild-type and azole-resistant *A. fumigatus* strains to lipophilic antifungal drugs. Kinetic studies with labeled posaconazole indicate that the increased susceptibility to antifungals is due to increased penetration of fungal cells by these agents. Both $Sph3_h$ and $PelA_h$ were nontoxic to mammalian cells and protected epithelial cells from *A. fumigatus*-induced damage for up to 24 h. Intratracheal delivery of $Sph3_h$ was well tolerated by mice and significantly reduced the fungal burden of immunocompromised mice infected with *A. fumigatus*. Our results suggest that glycoside hydrolases have the potential to be effective antibiofilm therapeutics that can mediate activity against evolutionarily diverse microorganisms.

Results

$Sph3_h$ Disrupts Preformed *A. fumigatus* Biofilms. Our previous work demonstrated that $Sph3_h$ from *A. fumigatus* and *Aspergillus clavatus* can hydrolyze both purified and cell wall-bound GAG on young hyphae (23). We therefore sought to determine whether the degradation of GAG by $Sph3_h$ could disrupt established *A. fumigatus* biofilms. Treatment with $Sph3_h$ for 1 h disrupted established *A. fumigatus* biofilms with an effective concentration for 50% activity (EC_{50}) of 0.45 ± 1.31 nM (Fig. 1*A*). Biofilm disruption was associated with a marked reduction in hyphae-associated GAG as detected by lectin staining (Fig. 1 *B* and *C*) and scanning electron microscopy (Fig. 1*D*). A catalytic variant $Sph3_h$ D166A, which does not mediate GAG hydrolysis (23), displayed a greater than 500-fold reduction in antibiofilm activity (Fig. 1*A*) and failed to mediate degradation of biofilm-associated GAG (Fig. 1 *B* and *C*). Collectively, these data suggest that biofilm disruption is mediated through the enzymatic hydrolysis of GAG.

To validate that fungal biofilm disruption by $Sph3_h$ is not restricted to the *A. fumigatus* laboratory strain Af293, the activity of $Sph3_h$ was evaluated against four clinical *A. fumigatus* isolates. $Sph3_h$ disrupted biofilms of all isolates tested at EC_{50} values <0.15 nM (Fig. S1). These results confirm the role of GAG in biofilm formation and indicate that $Sph3_h$ exhibits antibiofilm activity across a wide range of *A. fumigatus* strains.

The Bacterial Hydrolase $PelA_h$ Hydrolyses GAG and Disrupts Fungal Biofilms. Given that GAG and Pel are both cationic exopolysaccharides containing 1,4-linked GalNAc (14, 17), we hypothesized that $PelA_h$ might exhibit activity against GAG. Consistent with this hypothesis, an in vitro reducing sugar assay demonstrated that $PelA_h$ was capable of hydrolyzing purified GAG (Fig. S2). Furthermore, using the crystal violet biofilm assay, we found that $PelA_h$ disrupted *A. fumigatus* fungal biofilms with an EC_{50} value of 2.80 ± 1.14 nM (Fig. 2*A*). The treatment of *A. fumigatus* hyphae with $PelA_h$ also resulted in a reduction in the amount of cell wall-associated GAG (Fig. 2 *B*–*D*) as was observed with $Sph3_h$ treatment. The $PelA_h$ catalytic variant, $PelA_h$ E218A, which is markedly impaired in Pel hydrolysis and is inactive against *P. aeruginosa* biofilms (22), did not significantly hydrolyze GAG at concentrations as high as 12 μM (Fig. S2). Consistent with this observation, $PelA_h$ E218A was also several hundred-fold less active against *A. fumigatus* biofilms and did not degrade hyphae-associated GAG (Fig. 2 *B* and *C*). These results suggest that $PelA_h$ disrupts *A. fumigatus* biofilms through the hydrolysis of biofilm-associated GAG.

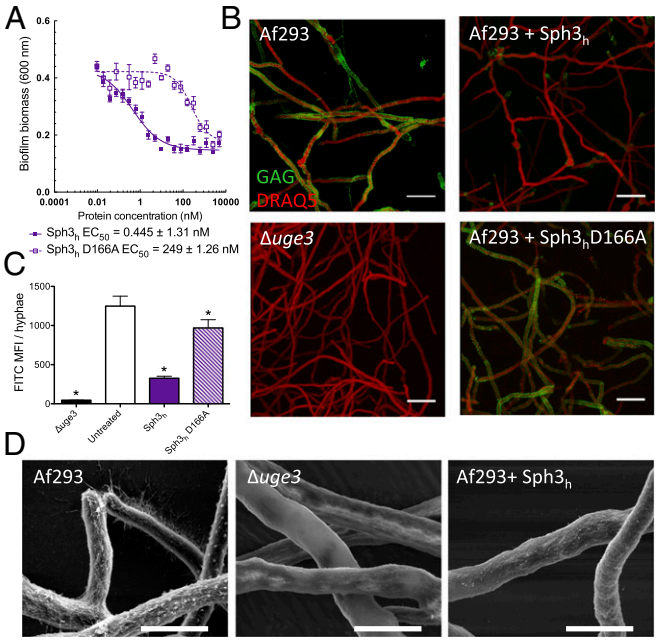

Fig. 1. Treatment with $Sph3_h$ disrupts *A. fumigatus* biofilms and degrades GAG on the surface of hyphae. (*A*) Crystal violet staining of established *A. fumigatus* biofilms treated with the indicated concentration of each hydrolase. Each data point represents the mean of $n = 20$ with error bars indicating SE. EC_{50} indicates the 50% effective concentration ± SE. (*B*) Effects of the indicated hydrolases on cell wall-associated GAG. Hyphae of the indicated strains grown in the absence of hydrolase treatment (*Left*) or following exposure to 0.5 μM of the indicated hydrolases (*Right*). Cell wall-associated GAG was visualized by using FITC-conjugated lectin staining (green) with DRAQ5 as a counterstain (red). The GAG-deficient Δ*uge3* mutant was included as a negative control. (*C*) Quantification of lectin-staining from *B*. Each data point represents the mean fluorescence intensity of at least seven hyphae with error bars indicating SE. * indicates a significant difference ($P < 0.05$) relative to the untreated *A. fumigatus* as determined by one-way ANOVA with Dunnett's multiple comparison test. (*D*) Scanning electron micrographs of hyphae of the indicated strains grown in the absence of hydrolases (*Left* and *Center*) and following exposure to 0.5 μM $Sph3_h$ (*Right*). (Scale bars: *B*, 20 μm; *D*, 5 μm.) MFI, mean fluorescence intensity.

$Sph3_h$ Binds Pel but Does Not Disrupt Established *P. aeruginosa* Biofilms. Given that $PelA_h$ can hydrolyze GAG and disrupt GAG-mediated biofilms, we hypothesized that $Sph3_h$ may exhibit activity against Pel and Pel-mediated biofilms. The inability to purify sufficient quantities of Pel precluded us from using it as a substrate. Therefore, to examine whether $Sph3_h$ was capable of hydrolyzing Pel, the enzyme was exogenously applied to biofilms produced by the Pel overproducing *P. aeruginosa* strain PAO1 Δ*wspF* Δ*psl* P_{BAD}*pel*. Treatment of these established biofilms with $Sph3_h$ did not affect levels of Pel within the biofilms as visualized by lectin staining (Fig. 3 *A* and *B*), nor did it reduce biofilm biomass, even at concentrations exceeding 10 μM (Fig. 3*C*).

Because $Sph3_h$ did not hydrolyze Pel within *P. aeruginosa* biofilms, we tested whether the enzyme was capable of recognizing and binding this polysaccharide. Using an ELISA-based

MICROBIOLOGY

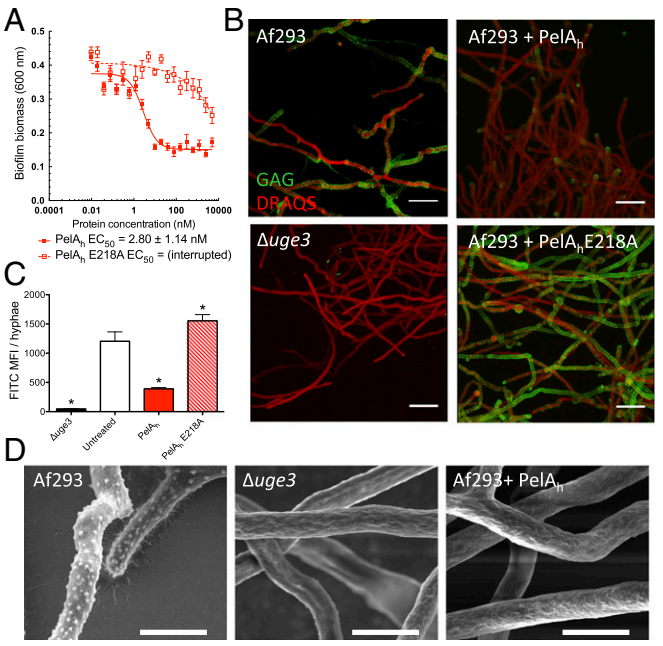

Fig. 2. PelA$_h$ disrupts *A. fumigatus* biofilms and degrades GAG. (*A*) Effects of PelA$_h$ on *A. fumigatus* biofilms. Crystal violet staining of established *A. fumigatus* biofilms treated with the indicated concentration of PelA$_h$ or PelA$_h$ E218A. Each data point represents the mean of $n = 20$ with error bars indicating SE. EC$_{50}$ reported ± SE. (*B*) Effects of the indicated hydrolases on cell wall-associated GAG. Established hyphae of the indicated strains were untreated (*Left*) or exposed to 0.5 μM of the indicated hydrolases (*Right*). Cell wall-associated GAG was detected by using FITC-conjugated lectin staining (green), with DRAQ5 as a counterstain (red). (*C*) Mean fluorescent intensity of lectin staining in *B*. Each data point represents the mean of at least seven hyphae with error bars indicating SE. The * indicates a significant difference ($P < 0.05$) relative to the untreated *A. fumigatus* as determined by one-way ANOVA with Dunnett's multiple comparison test. (*D*) Scanning electron micrographs of hyphae of the indicated strains grown in the absence of hydrolase treatment (*Left* and *Center*) or following treatment with 0.5 μM PelA$_h$ (*Right*). (Scale bars: *B*, 20 μm; *D*, 5 μm.) MFI, mean fluorescence intensity.

binding assay, we observed dose-dependent binding of Sph3$_h$ to culture supernatants from the Pel overproducing *P. aeruginosa* strain, but not from supernatants of the Pel-deficient strain PAO1 Δ*wspF* Δ*pel* Δ*psl* (Fig. 3*D*). These data suggest that the inability of Sph3$_h$ to disrupt Pel-mediated biofilms is likely a consequence of an inability to hydrolyze Pel rather than being unable to bind the polysaccharide. Dose-dependent binding of the inactive Sph3$_h$ D166A variant to GAG-containing culture supernatants was also observed (Fig. S3), suggesting that binding of hydrolases to exopolysaccharides is insufficient to disrupt established biofilms in the absence of enzymatic cleavage of the polymer.

Sph3$_h$ and PelA$_h$ Potentiate Antifungals by Enhancing Their Intracellular Penetration. The Pel polysaccharide enhances resistance to several antibiotics including aminoglycosides and colistin (22, 24, 25). Because biofilm formation by *A. fumigatus* is associated with increased resistance to a number of antifungal agents (26–28), we hypothesized that GAG may have an analogous function to Pel in enhancing resistance to antifungal agents. To test this hypothesis, we investigated whether Sph3$_h$ or PelA$_h$ could potentiate the activity of commonly used antifungal drugs. Treatment of established fungal biofilms with either enzyme resulted in a significant reduction in the MIC$_{50}$ of the azole posaconazole, the polyene amphotericin B, and the echinocandin caspofungin (Fig. 4*A*). Sph3$_h$ or PelA$_h$ treatment produced a similar increase in sensitivity to posaconazole for both azole-sensitive and azole-resistant strains of *A. fumigatus* (Fig. S4). Susceptibility to voriconazole, a smaller and more polar azole, was unaffected by treatment with either glycoside hydrolase (Fig. 4*A*). Because both posaconazole and voriconazole have the same intracellular target, these findings suggest that cationic GAG mediates antifungal resistance by hindering cellular uptake of large, nonpolar molecules such as posaconazole. To investigate this hypothesis, the effect of Sph3$_h$ on intracellular penetration of posaconazole was examined by using posaconazole conjugated to the fluorophore BODIPY (BDP-PCZ). Previous work has established that BDP-PCZ displays similar cellular and subcellular pharmacokinetics to unmodified posaconazole (29). Fluorometric studies revealed that Sph3$_h$ treatment resulted in higher accumulation of BDP-PCZ within *A. fumigatus* hyphae (Fig. 4*B*). This finding indicates that GAG protects *A. fumigatus* from the action of lipophilic antifungals by limiting their penetration into hyphae.

Recombinant Sph3$_h$ and PelA$_h$ Protect Epithelial Cells from Damage by *A. fumigatus*. *A. fumigatus* GAG-mediated adherence is required for *A. fumigatus* to damage A549 pulmonary epithelial cells in vitro (20). We therefore tested whether treatment with either Sph3$_h$ or PelA$_h$ could protect epithelial cells from fungal-induced injury by using an established chromium (^{51}Cr) release damage assay (30). We first established that the enzymes were not cytotoxic and that the addition of Sph3$_h$ or PelA$_h$ to uninfected A549 cell monolayers did not cause detectable cellular damage (Fig. S5*A*), a finding verified with the IMR-90 human

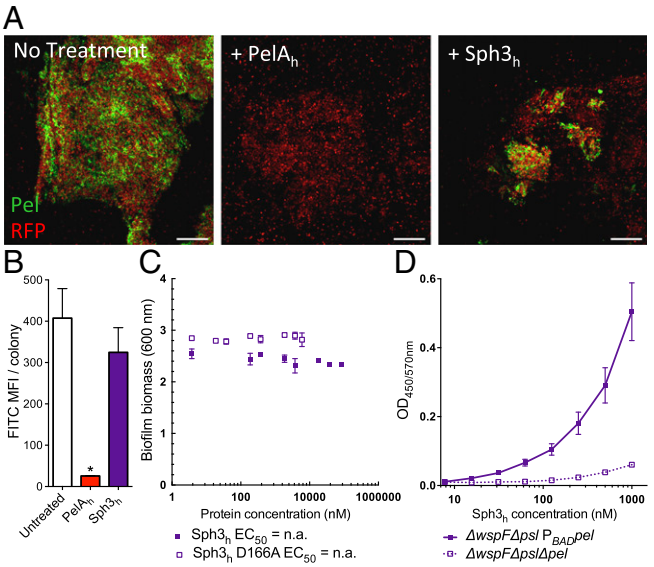

Fig. 3. Sph3$_h$ binds Pel but is inactive against established *P. aeruginosa* biofilm. (*A*) Established biofilms of RFP-producing *P. aeruginosa* overexpressing the Pel operon (red) were untreated (*Left*) or exposed to 0.5 μM of the indicated hydrolases (*Center* and *Right*). Biofilm-associated Pel was detected by using FITC-conjugated *Wisteria fluoribunda* lectin staining (green). (Scale bars: 20 μm.) (*B*) Mean fluorescent intensity of lectin staining in *A*. Each data point represents the mean of at least four *P. aeruginosa* colonies with error bars indicating SE. * indicates a significant difference ($P < 0.001$) relative to the untreated *P. aeruginosa* as determined by one-way ANOVA with Dunnett's multiple comparison test. (*C*) Effects of Sph3$_h$ on Pel-mediated *P. aeruginosa* biofilms. Crystal violet staining of established biofilms of *P. aeruginosa* overexpressing the Pel operon incubated with the indicated concentrations of Sph3$_h$ or Sph3$_h$ D166A. Each data point represents the mean of $n = 3$ with error bars indicating SE. EC$_{50}$ reported ± SE. (*D*) Sph3$_h$ binding to Pel polysaccharide. Microtiter plates were coated with culture supernatants of the indicated *P. aeruginosa* strains and the binding of Sph3$_h$ was determined by using an anti-Sph3$_h$ antibody. Each data point represents the mean of three independent experiments with error bars indicating SE. MFI, mean fluorescence intensity.

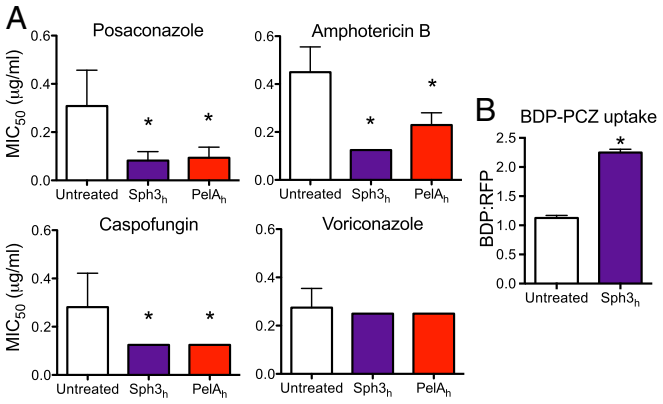

Fig. 4. Glycoside hydrolases increase sensitivity of *A. fumigatus* to antifungal agents. (*A*) Established biofilms of wild-type *A. fumigatus* strain Af293 were treated with the indicated concentrations of antifungals with or without 0.5 μM of the indicated hydrolase and the viability of the resulting biofilms was then measured by using the XTT metabolic assay. Susceptibility to antifungals was quantified by determining the antifungal concentration resulting in a 50% reduction in fungal metabolic activity (MIC_{50}) compared with untreated controls. Bars represent the mean of at least $n = 4$ with error bars indicating SE. (*B*) Effects of hydrolase therapy on antifungal uptake. *A. fumigatus* hyphae were treated with 1 μM $Sph3_h$ then exposed to 2 μg/mL BDP-PCZ. Uptake of BDP-PCZ was quantified via fluorometry. Each bar represents the mean of three independent experiments with error bars indicating SE. The * indicates a significant difference ($P < 0.05$) relative to untreated control samples as determined by one-way ANOVA with Dunnett's multiple comparison test in *A*, or two-tailed Student's *t* test in *B*. MIC, minimum inhibitory concentration.

lung fibroblast cell line (Fig. S5*B*). These data are consistent with the lack of cytotoxicity previously reported for $PelA_h$ (22). Next, we assessed whether $Sph3_h$ or $PelA_h$ were able to protect A549 cell monolayers from damage by *A. fumigatus*. $Sph3_h$ significantly reduced epithelial cell injury for up to 24 h (Fig. 5*A*). Treatment with $PelA_h$ also protected epithelial cells from *A. fumigatus*-induced damage (Fig. 5*A*). The protective effect of $PelA_h$ was shorter than that observed with $Sph3_h$, and was lost before 24 h of treatment. The addition of protease inhibitors extended $PelA_h$-mediated epithelial cell protection to 24 h (Fig. S5*C*), suggesting that the decrease in $PelA_h$-mediated protection was likely due to proteolytic degradation of the recombinant protein. Epithelial cell protection was not observed with the catalytic variants, $PelA_h$ E218A or $Sph3_h$ D166A, suggesting that the hydrolytic activity of the enzymes is required for protection (Fig. 5*A*).

Intratracheal $Sph3_h$ Is Well Tolerated, and Attenuates Fungal Virulence in an Immunocompromised Mouse Model of Pulmonary Aspergillosis. Given the ability of $Sph3_h$ to protect epithelial cells for more than 24 h, this hydrolase was selected for evaluation in vivo. BALB/c mice treated intratracheally with doses up to 500 μg of $Sph3_h$ exhibited no signs of stress, weight loss, or change in body temperature after treatment (Fig. S6 *A* and *B*). Additionally, no significant increase in pulmonary injury or inflammation between treated and untreated mice were observed as measured by bronchoalveolar lavage lactate dehydrogenase activity and total pulmonary leukocyte populations (Fig. 5*B* and Fig. S6*C*). Collectively these results suggest that a single intratracheal dose of $Sph3_h$ is well tolerated by mice.

To determine the ability of $Sph3_h$ to attenuate virulence of *A. fumigatus*, neutropenic BALB/c mice were infected intratracheally with *A. fumigatus* conidia with or without the coadministration of 500 μg of $Sph3_h$. Four days after infection, mice infected with *A. fumigatus* and treated with $Sph3_h$ had a significantly lower pulmonary fungal burden to untreated, infected mice as measured by both fungal DNA (Fig. 5*C*) and pulmonary galactomannan content (Fig. S7). The fungal burden of the $Sph3_h$-treated mice was similar to that observed with mice infected with the GAG-deficient hypovirulent strain Δ*uge3* (20). Consistent with the fungal burden data, histopathologic examination of lung sections revealed the presence of fungal lesions in untreated, infected mice, but no detectable lesions in the lungs of infected mice treated with $Sph3_h$, or those infected with conidia of the Δ*uge3* mutant (Fig. 5*D*). These findings suggest that $Sph3_h$-mediated degradation of GAG can limit the growth of *A. fumigatus* in vivo, to the same degree as is observed with GAG-deficient organisms.

Discussion

In this study, we demonstrate that the fungal glycoside hydrolase $Sph3_h$ is able to degrade preformed *A. fumigatus* biofilms. This study is an example of the use of a glycoside hydrolase to disrupt a fungal biofilm. Further, we establish that the glycoside hydrolase $PelA_h$ displays activity against biofilms formed by organisms

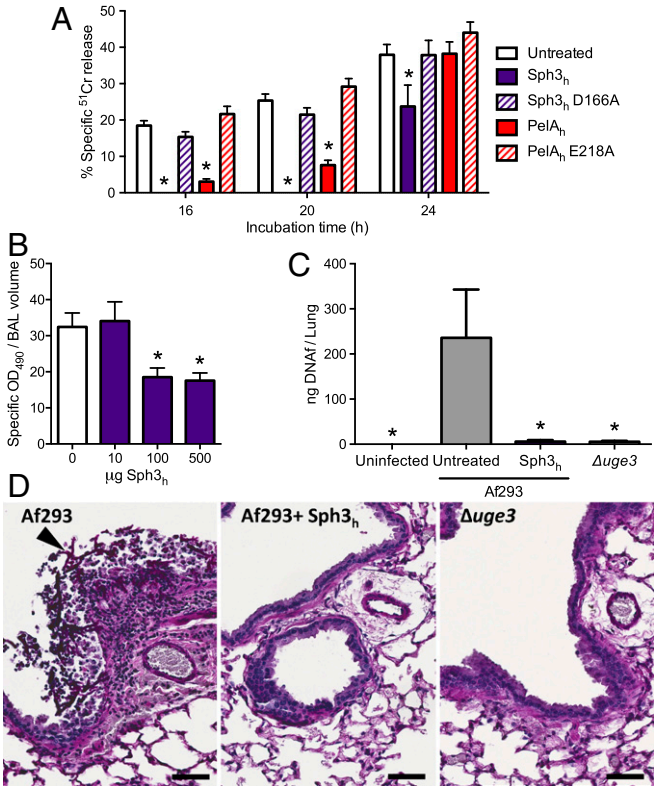

Fig. 5. Effects of hydrolases on *A. fumigatus*-induced airway epithelial cell damage and in vivo pulmonary infection. (*A*) ^{51}Cr-loaded A549 pulmonary epithelial cells were incubated with conidia of wild-type *A. fumigatus* in the presence or absence of 0.5 μM concentrations of the indicated hydrolases. Epithelial cell damage was determined by measurement of the amount of ^{51}Cr released into supernatant at the indicated time points. Each bar represents the mean of at least five independent experiments performed in duplicate with error bars indicating SE. (*B*) Pulmonary injury as measured by lactose dehydrogenase activity of the bronchoalveolar lavage fluid from BALB/c mice treated intratracheally or not with the indicated quantities of $Sph3_h$ and killed 7 d after treatment. Data represents the mean of at least $n = 5$, with error bars representing SE. (*C*) Fungal burden of neutropenic mice as determined by quantitative PCR following 4 d of infection with the indicated *A. fumigatus* strain with or without treatment with a single dose of 500 μg of $Sph3_h$. Data represents the mean of at least $n = 12$, from two independent experiments with error bars indicating SE. The * indicate a significant difference $P < 0.01$ for *A* and *C*, and < 0.05 for *B*, relative to untreated controls by using a two-way ANOVA for *A* and a one-way ANOVA for *B* and *C* with a Dunnett's multiple comparison test. (*D*) Histopathological analysis of lung sections of mice from *C* stained with periodic acid Schiff reagent. Arrow indicates hyphal lesion. (Scale bars: 20 μm.)

MICROBIOLOGY

across different microbial kingdoms. Both glycoside hydrolases potentiated the penetration and activity of antifungal agents in vitro, exhibited no toxicity against mammalian cells, and protected epithelial cells from *A. fumigatus*-induced damage. Pulmonary administration of $Sph3_h$ was well tolerated and limited fungal growth in an immunocompromised mouse model, suggesting that these enzymes are promising therapeutic agents for the treatment of fungal disease.

The mechanism by which the biofilm matrix enhances *A. fumigatus* resistance to antifungals is poorly understood. The effect of hydrolase treatment on the antifungal sensitivity of *A. fumigatus* provides some insight into this question and establishes a role for GAG in biofilm-associated antifungal resistance. Multiple observations suggest that GAG enhances antifungal resistance by acting as a barrier to antifungal penetration of hyphae. First, glycoside hydrolase degradation of GAG enhanced the activity of multiple antifungals with different mechanisms of action. Second, the activity of posaconazole, but not voriconazole, was enhanced although both azoles target the same enzyme, CYP51A. These hydrolases also display similar activity against azole-resistant and azole-sensitive strains. The cationic nature of GAG may explain the differential effects on voriconazole compared with other antifungals. The GAG barrier would be predicted to be most effective against large, lipophilic or cationic antimicrobial agents, and thus therapeutic hydrolases may be most effective as adjuvants for lipophilic antifungals. Previous studies have reported that the enzymatic degradation of neutral α-glucans of *A. fumigatus* did not enhance susceptibility to antifungals (31), further supporting our hypothesis that exopolysaccharide charge plays a role in mediating antibiotic resistance. Similarly, hydrolysis of cationic Pel exopolysaccharide by $PelA_h$ enhances the activity of the polycationic antibacterial colistin (22). Interestingly, degradation of biofilm-associated extracellular DNA (eDNA) has been reported to enhance *A. fumigatus* susceptibility to caspofungin and amphotericin B, although the effects on posaconazole and voriconazole susceptibility were not reported in the study (26). Recent work has suggested that Pel anchors eDNA within *P. aeruginosa* biofilms through charge–charge interactions (17). Given the similarities between Pel and GAG, it is possible that GAG-mediated binding of eDNA may also contribute to enhancing antifungal resistance.

The results of these studies add to an emerging body of evidence that fungal biofilms share structural (32–35) and functional (26, 36, 37) similarity with those formed by pathogenic bacteria. The finding that glycoside hydrolases can display activity against the exopolysaccharides and biofilms of both fungi and bacteria provides evidence that these similarities could potentially be exploited for the development of therapeutics active against both organisms. Additionally, the similarity between the exopolysaccharides of *P. aeruginosa* and *A. fumigatus*, coupled with the interspecies activity of their glycoside hydrolases, suggest the intriguing possibility that exopolysaccharide interactions may occur between organisms during multispecies biofilm formation. Cocolonization with *P. aeruginosa* and *A. fumigatus* is not uncommon in patients with chronic pulmonary disease such as cystic fibrosis (38). Although studies of the formation of mixed fungal-bacterial biofilms during pulmonary infection are limited, a recent study of patients with chronic lung disease reported that antibacterial therapy for *P. aeruginosa* was associated with a reduction in fungal colonization, suggesting the possibility of microbial cooperation (39). Further studies examining the role of cross-species exopolysaccharide and exopolysaccharide-modifying enzyme interactions are required to establish a role for cooperative biofilm interactions in pulmonary disease.

Whereas $PelA_h$ exhibited cross-species activity and disrupted preformed fungal biofilms, $Sph3_h$ bound Pel, but was unable to disrupt established Pel-mediated biofilms. This difference in activity may reflect differences in the composition or conformation of each polysaccharide because GAG is a heteropolymer of GalNAc and galactose whereas Pel is comprised of GalNAc and GlcNAc. It is likely that these differences influence the ability of $Sph3_h$ and $PelA_h$ to hydrolyze the polymer. The inability of $Sph3_h$ to degrade preformed *P. aeruginosa* biofilms may suggest that mature Pel adopts a configuration or undergoes postsynthetic modification that renders it incompatible with the catalytic active site of $Sph3_h$ and resistant to cleavage. Detailed studies of these enzymes to determine the mechanisms underlying their differential activity against Pel will require purified polysaccharide, which is not available.

Both $Sph3_h$ and $PelA_h$ were found to be noncytotoxic, and a single dose of intratracheal $Sph3_h$ was well tolerated by BALB/c mice. Coadministration of $Sph3_h$ with wild-type conidia to neutropenic mice greatly reduced fungal outgrowth within the lungs of these mice. Together these results provide proof-of-concept that the glycoside hydrolases can be used to improve the outcome of fungal infection, with minimal side effects and toxicity. These findings will pave the way for future work to evaluate the utility of these agents as antifungal therapeutics including detailed pharmacokinetic and toxicity studies, and the evaluation of these enzymes for the treatment of established fungal infections alone and in combination therapy with lipophilic antifungal agents such as posaconazole or amphotericin B.

Methods

Strains and Culture Conditions. Strains used in this study are detailed in Table S1, and detailed culture conditions are described in *SI Methods*.

Recombinant Hydrolase Expression and Purification. Hydrolases were expressed and purified as described (22, 23).

Treatment of *A. fumigatus* with Glycoside Hydrolases. To visualize the effects of hydrolases on cell wall-associated GAG, hyphae were treated with recombinant hydrolases and stained with fluorescein-conjugated soybean agglutinin as described (23), with minor modifications. Hyphae were counterstained with a 1:1,000 dilution of DRAQ5 (eBioscience) in PBS for 5 min before paraformaldehyde (PFA) fixation. Complete image acquisition and processing methods can be found in *SI Methods*. To study the effects of hydrolases on biofilms, 10^4 conidia were grown in Brian media in polystyrene, 96-well plates for 19 h at 37 °C and 5% CO_2 and then treated with the indicated concentration of glycoside hydrolase in PBS for 1 h at room temperature. Biofilms were then gently washed, stained with 0.1% (wt/vol) crystal violet and destained with 100% ethanol for 10 min. The optical density of the destain fluid was measured at 600 nm (OD_{600}).

Scanning Electron Microscopy. Complete sample preparation and processing methods are detailed in *SI Methods*.

Treatment of *P. aeruginosa* with Glycoside Hydrolases. For biofilm disruption, static *P. aeruginosa* cultures were grown for 22 h at 30 °C, at which point the planktonic cells were aspirated, and LB + 0.5% arabinose + 0.5 μM glycoside hydrolase was added for an additional 3 h. For the detection of Pel, samples were incubated with 30 μg/mL fluorescein-conjugated *Wisteria fluoribunda* lectin for 2 h at 4 °C, fixed with 8% (wt/vol) PFA for 20 min at 4 °C and imaged as detailed in *SI Methods*. The ability of hydrolases to disrupt established biofilms were studied as described (22).

Quantification of Hydrolase Binding to Culture Supernatants. Details of culture supernatant production and hydrolase binding quantification can be found in *SI Methods*.

Effects of Glycoside Hydrolases on Antifungal Susceptibility of *A. fumigatus*. Fungal biofilms were prepared in tissue culture treated with 24-well plates in RPMI medium 1640 (Life Technology) buffered with Mops [3-(*N*-Morpholino) Propane-Sulfonic Acid] (RPMI-Mops) (Fisher) for 9 h at 37 °C, 5% CO_2. Serial dilutions of antifungal compounds with or without 0.5 μM $Sph3_h$ or $PelA_h$ were added to wells, and the plates were incubated at 37 °C and 5% CO_2 for 15 h. Fungal viability was measured by using the sodium 3′-{1-[(phenylamino)-carbony]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) metabolic assay as described (40). The concentration of antifungal resulting in a 50% decrease in viability (MIC_{50}) was used as a measure of antifungal effect.

Fluorometric Quantification of Hyphal Uptake of BDP-PCZ. A total of 2.5×10^4 conidia of red fluorescent protein (RFP)-expressing *A. fumigatus* were grown

in each well of a 96-well black, clear-bottom plate for 8 h at 37 °C, 5% CO_2. Hyphae were treated with 1 μM $Sph3_h$ in PBS for 90 min at 37 °C, 5% CO_2 then exposed to 2 μg/mL BDP-PCZ for 10 min. The plate was then read by using an Infinite M1000 fluorescent plate reader with excitation wavelengths of 532 and 488 nm for RFP and BDP-PCZ, respectively. Background fluorescence was subtracted from both RFP and BDP-PCZ signals, and the BDP-PCZ signal was then normalized to total RFP fluorescence for each well.

Effects of Glycoside Hydrolases on *A. fumigatus*-Induced Epithelial Cell Damage. A549 pulmonary epithelial cell damage by *A. fumigatus* was tested by using the ^{51}Cr release assay as described (20, 30). Recombinant hydrolases were added to the A549 cultures at the time of infection at a final concentration of 0.5 μM.

Characterization of Pulmonary Damage by $Sph3_h$. All procedures involving mice were approved by the Animal Care Committees of the McGill University Health Centre. Female BALB/c mice 5–6 wk of age were anesthetized with isoflurane and administered a single endotracheal injection of 500 μg of $Sph3_h$ in 50 μL PBS and monitored daily for 7 d. Mice were then euthanized by CO_2 overdose, and their airways lavaged with 1 mL of PBS that was administered and collected through a needle inserted in the trachea. A total of two lavages were performed and pooled. The presence of LDH in the BAL fluid was used as an indicator of pulmonary damage; LDH activity was measured in the fluid by using a commercial assay (Promega), as per manufacturer's instructions.

Effects of $Sph3_h$ in a Severely Immunocompromised Mouse Model of Invasive Pulmonary Aspergillosis. Mice were immunosuppressed with cortisone acetate and cyclophosphamide as described (20, 41). Mice were infected with an endotracheal injection of 5×10^3 *A. fumigatus* conidia, resuspended in either PBS alone, or in combination with 500 μg of $Sph3_h$. Mice were monitored daily, and moribund animal were euthanized. At 4 d after infection, mice were euthanized and their lungs were harvested. For fungal burden analysis, lungs were homogenized in 5 mL of PBS containing protease inhibitor mixture (Roche), and aliquots were stored at −80 °C until use. Pulmonary fungal burden was determined as described (15), and as detailed in *SI Methods*.

ACKNOWLEDGMENTS. Research described in this paper is supported by Canadian Institutes of Health Research (CIHR) Grants 81361 (to P.L.H. and D.C.S.), 123306 (to D.C.S.), and 43998 (to P.L.H.) and Cystic Fibrosis Canada (CFC) (D.C.S. and P.L.H). B.D.S. has been supported by graduate scholarships from CFC and CIHR. N.C.B. has been supported in part by graduate scholarships from the Natural Sciences and Engineering Research Council of Canada; Mary H. Beatty and Dr. James A. and Connie P. Dickson Scholarships from the University of Toronto; CFC; and The Hospital for Sick Children. P.B. has been supported in part by a CFC Postdoctoral Fellowship and a Banting Fellowship from CIHR. P.L.H. is the recipient of a Canada Research Chair. D.C.S. is supported by a Chercheur-Boursier Award from the Fonds de Recherche Quebec Santé.

1. Bajwa S, Kulshrestha A (2013) Fungal infections in intensive care unit: Challenges in diagnosis and management. *Ann Med Health Sci Res* 3:238–244.
2. Peleg AY, Hooper DC (2010) Hospital-acquired infections due to gram-negative bacteria. *N Engl J Med* 362:1804–1813.
3. Vincent JL, et al.; EPIC International Advisory Committee (1995) The prevalence of nosocomial infection in intensive care units in Europe. Results of the European Prevalence of Infection in Intensive Care (EPIC) Study. *JAMA* 274:639–644.
4. Maltezou HC (2009) Metallo-β-lactamases in Gram-negative bacteria: Introducing the era of pan-resistance? *Int J Antimicrob Agents* 33:405.e1–405.e7.
5. Brown GD, et al. (2012) Hidden killers: Human fungal infections. *Sci Transl Med* 4:165rv13.
6. Verweij PE, et al. (2015) International expert opinion on the management of infection caused by azole-resistant Aspergillus fumigatus. *Drug Resist Updat* 21-22:30–40.
7. Mann EE, Wozniak DJ (2012) Pseudomonas biofilm matrix composition and niche biology. *FEMS Microbiol Rev* 36:893–916.
8. Gravelat FN, et al. (2010) Aspergillus fumigatus MedA governs adherence, host cell interactions and virulence. *Cell Microbiol* 12:473–488.
9. Sheppard DC (2011) Molecular mechanism of Aspergillus fumigatus adherence to host constituents. *Curr Opin Microbiol* 14:375–379.
10. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 35:322–332.
11. Seidler MJ, Salvenmoser S, Müller FMC (2008) Aspergillus fumigatus forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. *Antimicrob Agents Chemother* 52:4130–4136.
12. Alhede M, Bjarnsholt T, Givskov M, Alhede M (2014) Pseudomonas aeruginosa biofilms: Mechanisms of immune evasion. *Adv Appl Microbiol* 86:1–40.
13. Lee MJ, et al. (2015) The fungal exopolysaccharide galactosaminogalactan mediates virulence by enhancing resistance to neutrophil extracellular traps. *PLoS Pathog* 11: e1005187.
14. Fontaine T, et al. (2011) Galactosaminogalactan, a new immunosuppressive polysaccharide of Aspergillus fumigatus. *PLoS Pathog* 7:e1002372.
15. Lee MJ, et al. (2016) Deacetylation of fungal exopolysaccharide mediates adhesion and biofilm formation. *MBio* 7:e00252–16.
16. Franklin MJ, Nivens DE, Weadge JT, Howell PL (2011) Biosynthesis of the Pseudomonas aeruginosa extracellular polysaccharides, Alginate, Pel, and Psl. *Front Microbiol* 2:167.
17. Jennings LK, et al. (2015) Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the Pseudomonas aeruginosa biofilm matrix. *Proc Natl Acad Sci USA* 112:11353–11358.
18. Colvin KM, et al. (2012) The Pel and Psl polysaccharides provide Pseudomonas aeruginosa structural redundancy within the biofilm matrix. *Environ Microbiol* 14: 1913–1928.
19. Pritt B, O'Brien L, Winn W (2007) Mucoid Pseudomonas in cystic fibrosis. *Am J Clin Pathol* 128:32–34.
20. Gravelat FN, et al. (2013) Aspergillus galactosaminogalactan mediates adherence to host constituents and conceals hyphal β-glucan from the immune system. *PLoS Pathog* 9:e1003575.
21. Yang L, et al. (2012) Polysaccharides serve as scaffold of biofilms formed by mucoid Pseudomonas aeruginosa. *FEMS Immunol Med Microbiol* 65:366–376.
22. Baker P, et al. (2016) Exopolysaccharide biosynthetic glycoside hydrolases can be utilized to disrupt and prevent Pseudomonas aeruginosa biofilms. *Sci Adv* 2:e1501632.
23. Bamford NC, et al. (2015) Sph3 is a glycoside hydrolase required for the biosynthesis of galactosaminogalactan in Aspergillus fumigatus. *J Biol Chem* 290:27438–27450.
24. Khan W, et al. (2010) Aminoglycoside resistance of Pseudomonas aeruginosa biofilms modulated by extracellular polysaccharide. *Int Microbiol* 13:207–212.
25. Colvin KM, et al. (2011) The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa. *PLoS Pathog* 7:e1001264.
26. Rajendran R, et al. (2013) Extracellular DNA release acts as an antifungal resistance mechanism in mature Aspergillus fumigatus biofilms. *Eukaryot Cell* 12:420–429.
27. Rajendran R, et al. (2011) Azole resistance of Aspergillus fumigatus biofilms is partly associated with efflux pump activity. *Antimicrob Agents Chemother* 55:2092–2097.
28. Mowat E, et al. (2008) Phase-dependent antifungal activity against Aspergillus fumigatus developing multicellular filamentous biofilms. *J Antimicrob Chemother* 62: 1281–1284.
29. Campoli P, et al. (2013) Pharmacokinetics of posaconazole within epithelial cells and fungi: Insights into potential mechanisms of action during treatment and prophylaxis. *J Infect Dis* 208:1717–1728.
30. Lopes Bezerra LM, Filler SG (2004) Interactions of Aspergillus fumigatus with endothelial cells: Internalization, injury, and stimulation of tissue factor activity. *Blood* 103: 2143–2149.
31. Beauvais A, et al. (2007) An extracellular matrix glues together the aerial-grown hyphae of Aspergillus fumigatus. *Cell Microbiol* 9:1588–1600.
32. Martins M, et al. (2010) Presence of extracellular DNA in the Candida albicans biofilm matrix and its contribution to biofilms. *Mycopathologia* 169:323–331.
33. Krappmann S, Ramage G (2013) A sticky situation: Extracellular DNA shapes Aspergillus fumigatus biofilms. *Front Microbiol* 4:159.
34. Fuxman Bass JI, et al. (2010) Extracellular DNA: A major proinflammatory component of Pseudomonas aeruginosa biofilms. *J Immunol* 184:6386–6395.
35. Vilain S, Pretorius JM, Theron J, Brözel VS (2009) DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms. *Appl Environ Microbiol* 75:2861–2868.
36. Martins M, Henriques M, Lopez-Ribot JL, Oliveira R (2012) Addition of DNase improves the in vitro activity of antifungal drugs against Candida albicans biofilms. *Mycoses* 55:80–85.
37. Chiang WC, et al. (2013) Extracellular DNA shields against aminoglycosides in Pseudomonas aeruginosa biofilms. *Antimicrob Agents Chemother* 57:2352–2361.
38. Paugam A, et al. (2010) Characteristics and consequences of airway colonization by filamentous fungi in 201 adult patients with cystic fibrosis in France. *Med Mycol* 48 Suppl 1:S32–S36.
39. Baxter CG, et al. (2013) Intravenous antibiotics reduce the presence of Aspergillus in adult cystic fibrosis sputum. *Thorax* 68:652–657.
40. Pierce CG, et al. (2008) A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat Protoc* 3:1494–1500.
41. Sheppard DC, et al. (2004) Novel inhalational murine model of invasive pulmonary aspergillosis. *Antimicrob Agents Chemother* 48:1908–1911.
42. Brannon MK, et al. (2009) Pseudomonas aeruginosa Type III secretion system interacts with phagocytes to modulate systemic infection of zebrafish embryos. *Cell Microbiol* 11:755–768.