Adhesive properties of *Aspergillus fumigatus* biofilms probed by atomic force microscopy and effects of alginate lyase enzyme

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Summary

A. fumigatus biofilms represent a problematic clinical entity, especially because of their recalcitrance to antifungal drugs, which poses a number of therapeutic implications for invasive aspergillosis, the most difficult-to-treat Aspergillus-related disease. While the antibiofilm activities of amphotericin B (AMB) deoxycholate and its lipid formulations (e.g., liposomal AMB [LAMB]) are well documented, the effectiveness of these drugs in combination with nonantifungal agents is poorly understood.

In the present study, *in vitro* interactions between polyene antifungals Amphotericin (AMB) and liposomal Amphotericin (LAMB) with alginate lyase (AlgL), an enzyme degrading the polysaccharides produced as extracellular polymeric substances (EPSs) within the biofilm matrix, against *A. fumigatus* biofilms were evaluated by using atomic force microscopy (AFM). AFM was used to image and quantify the effects of AlgL-antifungal combinations on biofilm-growing hyphal cells.

AFM analysis showed that when *A. fumigatus* biofilms were treated with AlgL or polyene alone, as well as with their combination, both a reduction of hyphal thicknesses and an increase of adhesive forces were observed compared to the findings for untreated controls, probably owing to the different action by the enzyme or the antifungal compounds. Interestingly, marked physical changes were noticed in *A. fumigatus* biofilms exposed to the AlgL-antifungal combinations compared with the physical characteristics detected after exposure to the antifungal alone, indicating that AlgL may enhance the antibiofilm activity of both AMB and LAMB, perhaps by disrupting the hypha-embedding EPSs and thus facilitating the drugs to reach biofilm cells.

Our results suggest that a combination of AlgL and a polyene antifungal may prove to be a new therapeutic strategy for invasive aspergillosis.

Key words: *Aspergillus fumigatus*, atomic force microscopy, biofilms, alginate lyase, amphotericin.

Introduction

Aspergillus fumigatus (*A. fumigatus*) is a ubiquitous saprophytic mold that is often found in decaying materials, such as in compost piles or within the walls of old buildings. *A. fumigatus* is considered an opportunistic pathogen, primarily causing disease in immunocompromised patients. The most severe form is invasive aspergillosis (IA), and *A. fumigatus* is responsible for 90% of cases (Latge, 1999). Mortality rates of 70-90% are reported in haematopoietic stem cell transplant recipients, the most at-risk group (Denning, 1998). Despite improvements in the diagnosis and treatment of IA, the number of cases and severity of the disease have increased dramatically due to the sheer numbers of patients being subjected to increasingly powerful immunosuppressive therapies (Barnes and Marr, 2006).

A. fumigatus has the ability to filament within the lungs forming dense intertwined mycelial balls (Mowat *et al.*, 2008). Typical biofilm structures, aggregates of microorganisms in which cells adhere to each other on a surface embedded with-

in a self-produced extracellular matrix (ECM), are observed either in the alveoli (IA) or in an existing cavity (aspergilloma). Growth of *A. fumigatus* as a multicellular community is also found on the surface of indwelling catheters, by which the fungus can often gain access to the bloodstream causing fungemia. Studies by confocal and electron microscopy have shown that the mycelium of *A. fumigatus* growing on an agar surface is covered by the hydrophobic ECM that cohesively bonds hyphae into a contiguous sheath. The ECM, mainly composed of galactomannan, $\alpha_{1\text{-}3}$-glucans, melanin, and proteins including major antigens and hydrophobins (Beauvais *et al.*, 2007), was shown to surround hyphae growing in the lung of immunocompromised mice experimentally infected with *A. fumigatus* (Loussert *et al.*, 2010). Persister cells, interactions with polysaccharides of the extracellular matrix and ECM itself, acting as a barrier, contribute to reduced antifungal drug susceptibility.

Recent observations have consistently shown that all antifungal drugs are significantly less effective when *A. fumigatus* is grown as a biofilm than when it is grown in the planktonic state (Müller *et al*, 2011; Fiori *et al.*, 2011; Ramage *et al.*, 2012), presumably as a reflection of multiple resistance mechanisms, including the ECM, which would prevent drug diffusion by acting as a physical barrier. This could contribute to the overall mortality with IA, which remains high, despite the use of newer broad-spectrum antifungal agents and diagnostic adjuncts (Segal *et al.*, 2007).

Different studies characterized fungal and bacterial biofilms (Nunez *et al.*, 2005; Jonas *et al.*, 2007; Oh *et al.*, 2007) and the mechanisms involved in their adhesion to different surfaces (Beech *et al.*, 2002). Difficulties in accurate detection of the ECM over a generic substrate (i.e., glass, catheter, cells, etc.) are commonly experimented with a number of different biofilms and surfaces. Nevertheless, different studies showed that atomic force microscopy technique is able to detect the properties of *fungi* biofilm with a nanometer resolution (Papi *et al.*, 2012).

In the present study, we investigated the effects of combinations of Amphotericin B (AMB), a macrocyclic, polyene broad-spectrum antifungal agent, and its lipid preparation (LAMB) with an enzyme (Alginate Lyase, AlgL) against preformed *A. fumigatus* biofilms, in an attempt to instill new therapeutical strategies for IA. To detect the effects of AlgL on *A. fumigatus* biofilm we used atomic force microscopy (AFM), an emerged powerful technique for the analysis of microbial systems (Wright, 2010).

Materials and Methods

Fungal organisms, culture conditions, and inoculum preparation.

The *A. fumigatus* Af293 (ATCC MYA-4609, CBS 101355) type strain (28) and 31 clinical isolates were used throughout this study. All the isolates were retrieved from their frozen glycerol stocks and were streaked on fresh Sabouraud dextrose agar (Kima, Padua, Italy) plates, until good sporulation was achieved following incubation at 37°C. For all experiments, conidial suspensions in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) were obtained as described elsewhere (Fiori *et al.*, 2011; Pierce *et al.*, 2008) and used to prepare the inocula (see below).

Biofilm formation

A. fumigatus biofilms were grown statically for 24 h at 37°C on 13-mm-diameter glass coverslips (Bioscience Tools, San Diego,CA) placed into a standard 24-well cell culture plate (Thermo Scientific), by dispensing a cell inoculum prepared as described above into selected wells of the plate(s). After biofilm formation, the medium was aspirated and the plates were washed in sterile phosphate-buffered saline (PBS) to remove planktonic and/or nonadherent cells. Biofilm biomass was assessed as described elsewhere (Mowat *et al.*, 2008). Briefly, biofilms were stained with 0.5% (wt/vol) crystal violet solution for 5 min, rinsed with distilled water, and destained with 95% ethanol. The absorbance was measured at 490 nm with a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA) in order to determine the quantity of biological material produced (data not shown).

Antifungal and AlgL solutions.

Standard powders of the following antifungals were used: AMB (Sigma-Aldrich) and LAMB (Gilead Sciences, Milan, Italy). Their stock solutions were freshly prepared according to the manufacturers' guidelines. The AlgL from Flavobacterium species (28,000 U/g) was purchased as a pure substance from Sigma-Aldrich,

and a stock solution was freshly prepared in sterile PBS.

Determination of MIC

The MICs for planktonic cells (PMICs) were determined with the reference Clinical and Laboratory Standards Institute (CLSI) M38-A2 broth microdilution method using final drug concentrations that ranged from 0.008 to 8 g/mL for both the AMB and LAMB antifungals. Briefly, for each *A. fumigatus* isolate, a conidial inoculum was prepared in RPMI 1640 medium and quantified to achieve a final concentration of 0.4×10^4 to 5×10^4 conidia/mL. Following incubation of the microtiter plates for 48 h at 37°C, MIC (inhibitory concentration [IC]) endpoints were defined as the lowest drug concentrations that caused complete visible inhibition of growth compared with that of the drug-free growth control (Clinical and Laboratory Standards Institute, 2008). Testing of each isolate was performed in triplicate.

AFM imaging and force measurement

To further evaluate the effect of both AMB formulations in combination with AlgL, samples for AFM studies were prepared as previously described (Papi *et al.*, 2012). Briefly, 24-h-old *A. fumigatus* biofilms that were formed on glass coverslips as mentioned above were treated with AlgL (10 U/mL), AMB (4 μg/mL), and LAMB (4 μg/mL) or with their combinations at 37°C for 24 h. Samples were then rinsed gently with ultrapure water and air dried for AFM imaging and force spectroscopy by using a NanoWizard II atomic force microscope (JPK Instruments, Berlin, Germany) in conjunction with an optical microscope (Axio Observer; Carl Zeiss, Milan, Italy). Atomic force micrographs were collected in contact mode using Si_3N_4 cantilevers (CSC16; Mikromasch USA, San Jose, CA) with the manufacturer's quoted resonance frequencies of 10 kHz (range, 7 to 14 kHz) and force constants of 0.03 N/m (range, 0.01 to 0.08 N/m) in air. Scanning across the sample surface in the x and y directions was performed at a scan speed of 1 Hz. The images shown are typical results, and they were recorded in height mode. Moreover, to detect the effects of AlgL on ECM we build Root Mean Square (RMS) images for each sample. RMS image visualizes areas with high height local value variation. The RMS of deviations from the mean value of a circular neighbourhood of radius 2.5 pixels centered around each sample is calculated and displayed. To analyze the adhesion force over the biofilm surface, force curves were acquired by recording the cantilever deflection as a function of the vertical displacement of the samples (Papi *et al.*, 2012), in order to give the variation of the deflection signal per nanometer. During the force-distance measurement, the scanning rate in the z direction was performed at 1 Hz. A Gwyddion image viewer (http://gwyddion.net) was used to analyze high-resolution topographic images (500 by 500 lines per scan) of the surface changes (e.g., height [in nanometers]), as well as force-distance measurements over the sample surface. Values were averaged from force curves collected in triplicate at 10 different points on the surface of mature biofilms for five areas per sample from each experimental condition.

Results

In order to examine the substructural effects of AlgL when tested in combination with AMB or LAMB on glass-formed *A. fumigatus* Af293 biofilms we carried out an AFM study involving imaging and adhesion measurements. Therefore, five treatment groups were studied: (i) AlgL at 10 U/mL (AlgL-10), (ii) AMB at 4 μg/mL (AMB-4), (iii) LAMB at 4 μg/mL (LAMB-4), (iv) AMB-4 plus AlgL-10, and (v) LAMB-4 plus AlgL-10. Representative AFM images of biofilm-embedded hyphae scanned in air are shown in Figure 1. Treated hyphae exhibited little variation in morphology compared with the morphology of those that were untreated, in spite of differences in their texture features. In Figure 1 (middle), we show representative magnification images of ECM. We observe when the Af293 biofilms were treated with AlgL alone and in combination with AMB or LAMB (samples from groups i, iv, and v) the peculiar mesh size, visible in untreated sample (A), totally disappear. Moreover, Alg + LAMB sample (Figure 1D) shows the presence of liposomal particle, as expected, on hyphae and external surfaces. These differences are also highlighted in RMS images (Figure 1-bottom)

In Figure 2A, it was displayed the plot of hyphal height data from all five treatment groups. Interestingly, although there was a significant ($P<0.01$) difference between AMB and LAMB in their effects on hyphae, the presence of AlgL sus-

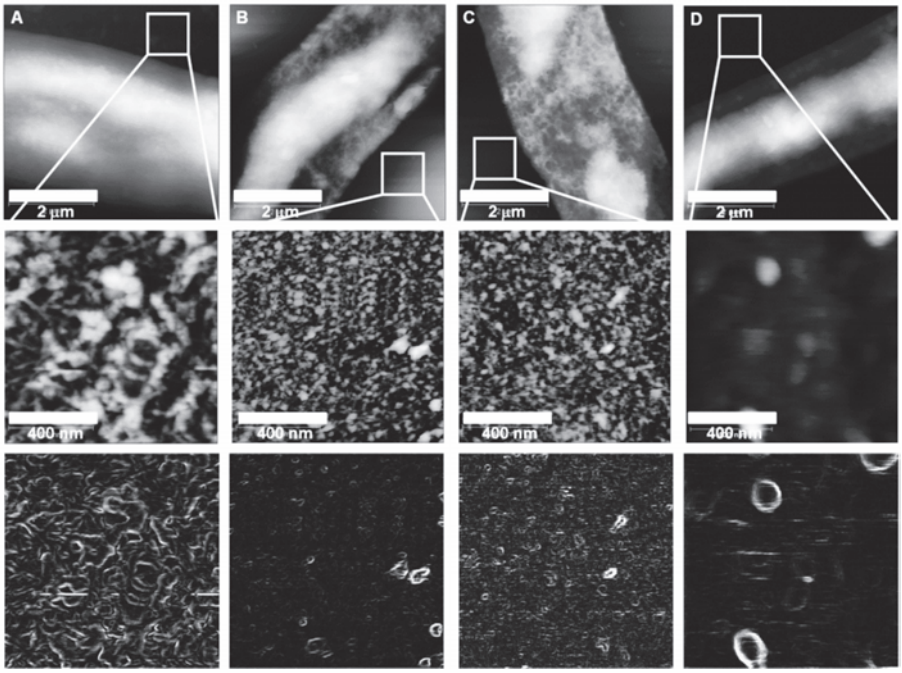

Figure 1. Atomic force micrographs of mature *A. fumigatus* Af293 biofilms imaged before treatment (A) and after treatment with AlgL (B), AlgL-AMB (C), or AlgL-LAMB (D). Topographic images (top) show hyphae from each experimental condition, as indicated, after they were grown as sessile cells for 24 h at 37°C in static and aerial environments. In the middle, magnification images (Size area 1µm^2) reveal more details in ECM biofilm texture. Finally, root mean square (RMS) images (bottom) of magnification images (Size area 1 µm^2) depict clear differences in mesh size structure between treated (B to D) and untreated control (A) samples.

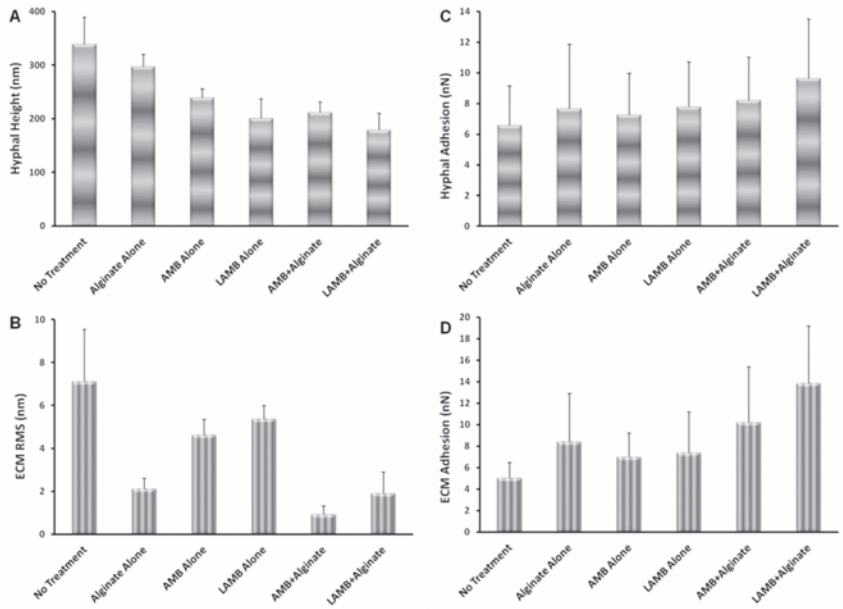

Figure 2. Biophysical properties of untreated 24-h-old *A. fumigatus* Af293 biofilms in RPMI 1640 medium and of biofilms treated for a further 24 h with AlgL or a polyene antifungal (AMB or LAMB) alone and with the AlgL-antifungal combinations, as measured by AFM. Averages of hyphal height (A), ECM-RMS (B) and adhesion force measured on hyphae surface (C) and ECM (D) from three independent experiments are shown. Error bars indicate the standard errors of the measurements.

tained such a difference, while significantly ($P<0.01$) improving the antihyphal activity of both AMB and LAMB compared with that of each antifungal alone. Moreover, as showed in Figure 2B, the effects of AlgL are prominent on ECM where the hole formation, due to enzyme, reflects upon an increase of RMS. The combination of AlgL and LAMB produces a statistically significant enhancement ($P<0.001$) of RMS value indicating a synergistic effect of both. Moreover, as attested to by adhesion force microscopy measurements, we found that the amount of adhesive values was significantly increased for all Af293 biofilms treated with AlgL ($P<0.01$) or antifungal drugs (AMB or LAMB; $P<0.05$), as well as with the AlgL and antifungal drug (AMB or LAMB) combinations ($P<0.001$), compared to that for untreated biofilms as measured on ECM (Figure 2C) and hyphae surface (Figure 2D). However, noticeable increases were especially seen for samples from treatment groups i (AlgL alone), iv (AlgL plus AMB), and v (AlgL plus LAMB). Once again, AlgL significantly potentiated the AMB ($P<0.05$) and LAMB ($P<0.01$) effects, and this finding was markedly evidenced for LAMB rather than for AMB, despite a slight difference between the antifungal drugs when used alone that favoured LAMB, but not significantly (Figure 2C-D).

These results suggest that the loss of hydrophobicity subsequent to enzymatic treatment diminishes the entrapment of AMB (and LAMB) in the ECM, thus accounting for the enhanced reduction of hyphal thickness in Af293 biofilms treated with the AlgL-antifungal combination.

Discussion

A. fumigatus is now a leading fungal pathogen, and one of the most significant opportunistic fungi in a range of patient groups including cystic fibrosis patients, HIV-positive patients and other immuno-compromised individuals (Cimon *et al.*, 2001; Singh, 2005). These infections are typified by intricate networks of hyphae that develop from inhaled conidia (Shibuya *et al.*, 2004).

The colony surface of *A. fumigatus* revealed the presence of a hydrophobic matrix that cohesively bonds hyphae into a contiguous sheath. The reduced permeability of the extracellular matrix delays penetration of the drugs into the cells and enhance the resistance of *A. fumigatus* to antimicrobial agents.

In such a context, combined use of a conventional antifungal with AlgL may provide an effective therapeutic approach, although no experiment addressed the activity or longevity of the enzyme under our assay conditions. If AlgL is short-lived, repeated applications at lower concentrations might be significantly better than single higher doses. In a clinical setting, AlgL can be administered as an injectable alginate-dissolving solution with minimal toxicity *in vivo* for the controlled release of the therapeutic agent.

The concentrations required and the implications of AlgL in experimental IA have yet to be determined, and further *in vitro* studies are needed to test the ability of AlgL to prevent biofilm formation. However, by interfering with the *A. fumigatus* biofilm matrix, it is possible that AlgL maximized the efficacy of both AMB and LAMB, although we did not specifically evaluate this hypothesis. Testing a second antifungal in a different class in parallel would certainly have strengthened our observations, but our observations reinforce the idea of the importance of the biofilm matrix in antifungal resistance, pointing to the use of EPS-degrading enzymes as a promising strategy to improve the management of biofilm-associated infections.

In this study we used atomic force spectroscopy, allowing an accurate determination of the biofilm's hydrophilic characteristics by measuring the interaction force between the AFM tip and the outer samples surface with the resolution of the tip contact area of 10 nm, to detect AlgL effects in combination with classical antifungal drugs (AMB and LAMB). In particular, local hydrophilic and electrostatic *A. fumigatus* properties and the biofilm formation and lysis have been discriminated together with morphology and topography properties.

We demonstrated that AlgL can greatly reduce the extent of the *A. fumigatus* biofilm forming several fractures that allows the drug transit and thus enhances the antifungal drug effect on the cell walls. We feel that clinical practice will benefit for this approach, by quantifying biofilms formed on medical devices (i.e., catheters) or evaluating the *in vitro* antifungal drug susceptibility.

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