In Vitro Interaction between Alginate Lyase and Amphotericin B against Aspergillus fumigatus Biofilm Determined by Different Methods

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Aspergillus 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 polynye antifungals (AMB and LAMB) and 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 the checkerboard microdilution and the time-kill assays. Furthermore, atomic force microscopy (AFM) was used to image and quantify the effects of AlgL-antifungal combinations on biofilm-growing hyphal cells. On the basis of fractional inhibitory concentration index values, synergy was found between both AMB formulations and AlgL, and this finding was also confirmed by the time-kill test. Finally, 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 antifungals 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. Taken together, our results suggest that a combination of AlgL and a polyene antifungal may prove to be a new therapeutic strategy for invasive aspergillosis, while reinforcing the EPS as a valuable antibiofilm drug target.

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imilar to biofilm-forming bacteria or yeasts (1), Aspergillus fumigatus, the most prevalent airborne fungal pathogen (2), is now largely acknowledged to be an organism able to grow and develop as a multicellular community (3), in which the hyphae are cohesively bonded together by a hydrophobic extracellular matrix (ECM) (4), under the aerial and static conditions found by the fungus either in vitro or in vivo (5–9). This growth phenotype, which complies with the definition of a biofilm (10), may help A. fumigatus to colonize the host substratum and to resist phagocytic and antimicrobial attacks, mimicking the typical Candida albicans or bacterial biofilm (8, 11). 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 (4, 7, 9, 12, 13), presumably as a reflection of multiple resistance mechanisms, including the ECM, which would prevent drug diffusion by acting as a physical barrier (14). This could contribute to the overall mortality with invasive aspergillosis, which remains high, despite the use of newer broad-spectrum antifungal agents and diagnostic adjuncts (15).

Amphotericin B (AMB), a macrocyclic, polyene broad-spectrum antifungal agent, preferably given as a lipid preparation (i.e., liposomal AMB [LAMB]) (16), is currently used in clinical practice for the treatment of invasive aspergillosis (17). However, high concentrations of AMB (at least 8 μg/ml) are needed to inhibit hyphal clumps (6, 18) or mature mycelia in vitro (13), although AMB was seen to be significantly more effective against each phase of A. fumigatus biofilm development and to display more rapid effects than other antifungals, such as voriconazole or caspofungin (13). In contrast to AMB deoxycholate, unique efficacy against Candida (C. albicans and C. parapsilosis) biofilms grown on a bio-prosthetic model was displayed by lipid-formulated AMB (19), probably due to the higher doses of drug present in these formulations. In another study, LAMB at 0.5 μg/ml was able to eradicate a C. albicans biofilm in a continuous-flow catheter model, whereas fluconazole and caspofungin were less effective (20). This somewhat surprising, different behavior between formulations of the same antifungal compound, coupled with the paucity of studies comparing their activities against fungal pathogens other than Candida, has enlivened our interest in testing both AMB formulations on an A. fumigatus biofilm.
Newer treatment strategies that incorporate substances not classified as antimicrobials, such as enzymes (e.g., DNase and alginate lyase [AlgL]) or quorum-sensing inhibitors, appear to increase biofilm susceptibility to conventional antibiotics (21, 22). If it should also be true with antifungals, this would allow the therapeutic dosage of AMB to be reduced, which is an advantage, given the well-known side effects and toxicity of the drug (23) as well as the cost of LAMB therapy, which limits its large-scale application, despite fewer adverse events and less nephrotoxicity than AMB deoxystreptolactone (16). In this regard, it was shown that AlgL, a carbohydrate-active enzyme which degrades uronic acid-containing polysaccharides (24) such as the alginate produced in exopolymERIC substances (EPSs) by mucoid strains of *Pseudomonas aeruginosa*, enhances antibiotic killing of alginate-producing *P. aeruginosa* in biofilms (25). In a recent work by Alipour et al. (21), addition of AlgL reduced the minimum biofilm eradication concentrations of free (conventional) and liposome-encapsulated aminoglycosides for mucoid *P. aeruginosa* strain PA-489121, although there was no significant difference between the free and liposomal forms of the antibiotics tested. To our knowledge, alginate has not been identified in the *A. fumigatus* ECM, which is mainly composed of galactomannan and α-1,3-glucans (4). However, other polyuronates, such as polyglucuronate (glucuronan), are found in fungi as water-soluble polysaccharides that are commonly degraded by AlgL-like enzymes (26) but that could be attacked nonspecifically by the bacterial AlgL.

In the present study, we investigated the *in vitro* effects of combinations of both AMB and LAMB with AlgL against preformed *A. fumigatus* biofilms, in an attempt to clarify the intriguing issues mentioned above. Besides assessing the drug interaction by conventional methods, we used atomic force microscopy (AFM), a recently emerged powerful technique for the analysis of microbial systems (27), to further evaluate the extent of these effects.

**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 (29; M. Sanguinetti, unpublished data) 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 (Kima, Padua, Italy) plates, until good sporulation was achieved following incubation at 37°C for 24 h, the wells were washed and the metabolic activity of sessile *A. fumigatus* was determined as described elsewhere (12, 28) and used to prepare the inocula (see below).

Biofilm formation and quantification. *A. fumigatus* biofilms were grown statically for 24 h at 37°C on polystyrene, flat-bottom, 96-well microtiter plates (Thermo Scientific, Milan, Italy) or, only for AFM studies, 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 (6). 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 (30), using final drug concentrations that ranged from 0.008 to 8 μg/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 × 10^6 to 5 × 10^6 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 (30). Testing of each isolate was performed in triplicate.

The MICs for sessile (biofilm) cells (SMICs) were determined by a 96-well microtiter-based method as previously described (12) with an inoculum of 1 × 10^6 conidia/ml (6) and with AMB and LAMB concentrations ranging from 0.125 to 128 μg/ml. This method, originally developed for investigating yeast adhesion and susceptibility but subsequently adapted to *A. fumigatus* (6, 31), is based on the reduction of 2,3-bis[2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2H-tetrazolium-hydroxide] (XTT) by metabolically active sessile cells (28). Briefly, biofilm-containing wells were filled with RPMI 1640 medium which contained doubling concentrations of the antifungal drugs, whereas untreated biofilm wells (positive controls) were filled with the same medium without drugs for 24 h at 37°C. A 100-μl aliquot of the XTT salt solution (1 mg/ml in PBS) and 1 μM menadione solution (Sigma-Aldrich; prepared in 100% acetone) were added to each prewashed biofilm and to negative-control wells (for measurement of background XTT reduction levels). After measuring the amount of XTT formazan at 490 nm (12), SMICs were calculated and expressed as the lowest drug concentrations at which a 90% decrease in optical density (OD; i.e., a 90% reduction in fungal metabolism) in comparison with that for the biofilms formed in the absence of drug was detected. Testing of each isolate was performed in triplicate.

**Checkerboard susceptibility testing.** A two-dimensional (8-by-12) checkerboard array of serial concentrations of the test compounds was used in sterile 96-well, flat-bottom microtiter plates as the basis for calculation of a fractional inhibitory concentration (FIC) index (see below and references 32 and 33) for each of two designed combinations: AMB-AlgL and LAMB-AlgL. Isolates Af293 and CG261 (CG261 is one of the clinical isolates showing the highest antifungal SMICs) were tested in quadruplicate. The compounds were serially diluted 2-fold in the assay medium in order to obtain four times the final concentration, which ranged from 0.016 to 8 μg/ml for AMB or LAMB and from 0.06 to 40 U/ml for AlgL. The AlgL concentrations were chosen since a concentration of 30 U/ml had previously been shown to be sufficient to reduce biofilm formation in *A. fumigatus* (34) and was consistent with that reported by other authors (21). Wells were inoculated with 5 × 10^4 cells/ml of each isolate, and after incubation at 37°C for 48 h, the growth in the wells relative to the growth in the growth control well was visually recorded. The same checkerboard assay was used to test in quadruplicate the combinations of AMB-AlgL and LAMB-AlgL against the biofilms formed by Af293 and CG261 as described above, using concentration ranges of 0.125 to 128 μg/ml for the antifungals and 0.06 to 40 U/ml for AlgL, in accordance with the SMICs of the individual compounds. After incubation of the microtiter plates at 37°C for 24 h, the wells were washed and the metabolic activity of sessile cells was assessed using the XTT reduction assay, as specified above. The MICS of the compounds alone and of the isoeffective combinations were determined to be the first concentrations of compound showing no visual growth (optically clear well) (PMICs) or growth near 10% of the growth of an untreated control (SMICs). The drug interactions were analyzed using the nonparametric FIC index model based on the Loewe additivity zero-interaction theory (32). Synergy and antagonism were defined by FIC.
index values of ≤0.5 and >4, respectively, whereas values of >0.5 to 4.0 were indicative of no interaction (additivity/indifference) (35, 36).

**Time-kill curves.** To investigate the effect of concentration and exposure time on the activity of AlgL alone or in combination with AMB and LAMB, time-kill experiments were carried out using an adaptation of the methodology used by Zhou et al. (37). The 24-h-old AF293 and CG261 biofilms, developed in microtiter plates as described above, were incubated with defined concentrations of each compound (AlgL, 10 U/ml; AMB and LAMB, 8, 16, and 32 μg/ml) in RPMI 1640 medium under shaking (75 rpm) at 37°C. At different time points (0, 1, 2, 4, 8, 12, and 24 h) after incubation, the content of each well was aspirated and washed twice with PBS. Then, 100 μl of XTT-menadione solution was added. After 2 h of incubation, the XTT solution was transferred to the corresponding well of a new microtiter plate, and colorimetric changes were measured as described above. For each isolate tested, the experiments were performed in triplicate on two separate days.

**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 (34). Briefly, 24-h-old *A. fumigatus* biofilms that were formed on glass coverslips as described above, were incubated with defined concentrations of each compound (AlgL, 1.0 and 10 U/ml; AMB and LAMB, 8, 16, and 32 μg/ml) in RPMI 1640 medium under shaking (75 rpm) at 37°C. At different time points (0, 1, 2, 4, 8, 12, and 24 h) after incubation, the content of each well was aspirated and washed twice with PBS. Then, 100 μl of XTT-menadione solution was added. After 2 h of incubation, the XTT solution was transferred to the corresponding well of a new microtiter plate, and colorimetric changes were measured as described above. For each isolate tested, the experiments were performed in triplicate on two separate days.

**RESULTS AND DISCUSSION**

Biofilms, likely the preferred growth form of fungi (1), exhibit profound resistance to many antimicrobial agents, thereby representing an escalating problem in the context of human health (8). First, the activities of both AMB and LAMB against a series of clinical *A. fumigatus* isolates, mostly derived from cases of invasive pulmonary infection, were determined (29). As expected, elevated concentrations of AMB and LAMB were required to inhibit 90% of the isolates in the presence of biofilm (SMIC<sub>90</sub>, 16 μg/ml) compared to those required for the planktonic conditions (MIC<sub>90</sub> of AMB and LAMB, 0.5 and 0.125 μg/ml, respectively), as assessed by metabolic activity (XTT reduction assay) measurements (see Table S1 in the supplemental material). Then, one of these isolates, CG261, was used, together with the *A. fumigatus* reference strain AF293, to evaluate the potential of AlgL to enhance the *in vitro* activity of AMB and LAMB against fungal biofilms.

**Drug interaction studies.** We applied checkerboard microdilution analysis to investigate the effect of AlgL on the antifungal activities of AMB and LAMB against biofilm cells of *A. fumigatus* AF293 and CG261. As summarized in Table 1, SMICs of AMB and LAMB for sessile cells of both the isolates increased up to 32- and 128-fold, respectively, for AF293 (PMICs, 0.5 and 0.125 μg/ml, respectively) and up to 128- and 512-fold, respectively, for CG261 (PMICs, 0.25 and 0.0625 μg/ml, respectively). In contrast, SMIC values for AF293 and CG261 of AMB and AlgL tested in combination decreased to 8 μg/ml and 10 U/ml, respectively, whereas...
those of LAMB and AlgL decreased to 2 µg/ml and 10 U/ml, respectively, when the two compounds were combined. Also, the FIC indices for the two tested isolates ranged from 0.312 to 0.75 and from 1.5 to 3 when analyzed using the SMIC and the PMIC endpoints, respectively, and in no case was antagonism observed (Table 1). Unlike the planktonic cells, FIC index values for the biofilm cells of Af293 and CG261 were far less than 0.5, indicating synergism between AMB (or LAMB) and AlgL, with exception of the Af293 biofilm and the AMB-AlgL combination, for which additivity/indifference was observed (FIC index value, 0.75). Although cutoffs of 0.5 and 4 for synergy and antagonism, respectively, have arbitrarily been suggested for two-drug combinations, some researchers used a cutoff of 1 for defining additivity/indifference (33,38, 39), based on the Loewe theory. According to this, we could also claim synergistic activity for the combination of AMB and AlgL against the biofilm-grown Af293 (Table 1).

To date, very few studies have explored the in vitro effects of antifungals in combination with nonantifungal agents against Aspergillus species. In one study, Afeltra et al. (40) found potent synergistic interactions between seven nonantimicrobial membrane-active compounds (e.g., verapamil, fluphenazine) and itraconazole against clinical isolates of A. fumigatus resistant to itraconazole, but unfortunately, no evaluations were conducted on biofilm-grown fungal cells. Thus, and for the first time to our knowledge, the present combination experiments showed synergy against mature biofilms of two A. fumigatus isolates when AMB and LAMB were combined with a nonantifungal agent, AlgL. Although the FIC index assessment could be not fully appropriate in this situation, in which one of the agents is an enzyme with no effect on its own, some conclusions can be drawn. Data emerging from the C. albicans biofilm field demonstrate that AMB, facilitated by the amphiphilic nature, physically binds to β-1,3-glucans, a structural component of the fungal cell wall as well as the C. albicans biofilm ECM (41), and such a drug-sequestering ECM might hamper the polyene from reaching biofilm cells. So, it is plausible that the lowering of antifungal MICs for A. fumigatus biofilms in the presence of AlgL detected here could be ascribed to a nonspecific degradation of the ECM by the enzyme, thus enabling both AMB and LAMB to exert their antifungal action. Also, no antagonism was observed, this emphasizes the potential clinical importance of combining traditional antifungal agents with AlgL, provided that the range of safely achievable concentrations of AlgL is encompassed within the range of concentrations that were found in the present study to contribute to synergy.

**Time-kill studies.** To validate the results of the checkerboard microdilution analysis, preformed biofilms of A. fumigatus Af293
and CG261 were challenged with AMB and LAMB (at 0.5, 1, and 2 SMICs), alone or combined with defined concentrations of AlgL (1 and 10 U/ml), in order to monitor cell viability (i.e., the OD) versus time using the XTT assay, which measures the aforementioned fungal biomass-associated metabolic activity (31). As it was expected (13, 18), the effects of polyenes against *A. fumigatus* cells in biofilms were rapid, with the Af293 isolate exhibiting after 4 h of drug challenge 30.7% and 39.4% OD reductions at the lowest (8 μg/ml) and highest (32 μg/ml) concentrations of AMB, respectively. Notably, more pronounced effects were seen with LAMB, with which 38.5% and 59.7% OD reduction rates were achieved after 4 h of drug challenge (8 and 32 μg/ml, respectively) for the Af293 isolate (Fig. 1). Similar percentages were observed for the other isolate studied, CG261, but at more delayed times (8 h) of antifungal drug exposure (Fig. 2). However, >95% of the reduction in metabolic activity was obtained with Af293 at the highest concentrations (32 μg/ml for AMB and 16 μg/ml for LAMB), and there was still 10% activity 24 h after challenging CG261 with 32 μg/ml of both AMB and LAMB (Fig. 1 and 2), in agreement with previous studies (6, 13, 18). Conversely, AlgL did not affect the time-kill curve of *A. fumigatus* Af293 and CG261 biofilms challenged with 1 U/ml of the compound, while a minimal reduction in metabolic activity over the 24-h period was shown following exposure to 10 U/ml AlgL, with a maximal 26% metabolism reduction displayed after 24 h (Fig. 1 and 2). Interestingly, compared with the results obtained with the antifungal drugs alone, the combinations AMB-AlgL and LAMB-AlgL all consistently decreased the numbers of viable biofilm-associated fungal cells relative to those for the untreated controls. In particular, the combination AMB-AlgL (8 μg/ml and 10 U/ml) resulted in a reduction of percent viability that ranged from 44 (18.2% activity for the combination compared to 62.2% activity for the antifungal alone) at 8 h to 26.3 (4.7% activity for the combination compared to the antifungal alone) at 24 h for Af293 and from 29.5 (37.9% activity for the combination compared to 67.4% activity for the antifungal alone) at 8 h to 40.6 (4.7% activity for the combination compared to 39.9% activity for the antifungal alone) at 24 h for CG261. Similar results were obtained with the LAMB-AlgL (8 μg/ml and 10 U/ml) combination. Overall, these findings revealed that AlgL strikingly enhanced the antibiofilm effects of AMB and LAMB against *A. fumigatus*, especially for the Af293 isolate; in addition, they show that both AMB formulations reduced the metabolic activity of *A. fumigatus* sessile cells within a few hours of exposure, but the concentrations needed to initiate these effects were high and are not achievable in humans (42). As the toxicity of AMB is its major clinical draw-

![Time-kill curves of AlgL (1 and 10 U/ml) alone and in combination with AMB or LAMB at 8 μg/ml (A), 16 μg/ml (B), or 32 μg/ml (C) against biofilm cells of a clinical *A. fumigatus* isolate (CG261). The results are the averages of three replicates carried out on two separate occasions and are expressed as percent cellular viability determined by the XTT reduction assay. Error bars represent the standard errors of the means.](http://aac.asm.org/article-pdf/57/3/1279/8094880/1279.pdf)
back, despite some mitigation by the lipid-based formulations that have been developed (16), an antibiofilm-antifungal agent combination might indeed reduce the polyene dose required for therapy.

**AFM studies.** We carried out an AFM study involving imaging and force measurements, in order to examine the ultrastructural effects of AlgL when tested in combination with AMB or LAMB on glass-formed *A. fumigatus* Af293 biofilms. 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 Fig. 3. Treated hyphae exhibited little variation in morphology compared with the morphology of those that were untreated, in spite of differences in their texture features. As exemplified in Fig. 3 (bottom), decreases of height compared to the height of untreated biofilms (control samples) were noted when the Af293 biofilms were treated with AlgL alone and in combination with AMB or LAMB (samples from groups i, iv, and v). Similar differences in height images were detectable between the biofilms treated with AMB or LAMB alone (samples from groups ii and iii) and the untreated ones (data not shown). As presented in Fig. 4A, plots of overall height data from all five treatment groups showed that such differences reached statistical significance (*P* < 0.001), as was also the case for the differences observed when biofilms treated with AlgL or antifungal alone (samples from group i, ii, or iii) were compared to biofilms treated with the AlgL and antifungal drug combination (samples from group iv or v). Interestingly, although there was a significant (*P* < 0.01) difference between AMB and LAMB in their effects on hyphae, the presence of AlgL sustained such a difference, while significantly (*P* < 0.01) improving the antihyphal activity of both AMB and LAMB compared with that of each antifungal alone. As attested to by the force-distance curve measurements, we hence found that the amount of adhesive forces 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 (Fig. 4B). 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 favored LAMB, but not significantly (Fig. 4B).

Consistent with our prior assumption (34), these results suggest that the loss of hydrophobicity subsequent to enzymatic treat-
ment 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.

**Conclusions.** Despite many available antifungal drugs (43), there is yet no optimal therapy for invasive aspergillosis, and this has prompted many clinicians to utilize a combination antifungal approach to improve outcomes (44). 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 (http://www.faa.org/patents/app/20100080788). The concentrations required and the implications of AlgL in experimental invasive aspergillosis 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 of a second antifungal in a different class in parallel would certainly have strengthened our observations, but our observations per se 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.

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