Amphotericin B lipid complex exerts additive antifungal activity in combination with polymorphonuclear leucocytes against *Scedosporium prolificans* and *Scedosporium apiospermum*

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*Scedosporium prolificans* and *Scedosporium apiospermum* are resistant to most antifungal agents and cause refractory pulmonary and disseminated infections. The combined effects of deoxycholate amphotericin B, amphotericin B lipid complex and liposomal amphotericin B with human polymorphonuclear leucocytes (PMNs) in damaging hyphae of these fungi were evaluated by XTT assay. Amphotericin B lipid complex displayed a significant additive effect with PMNs against both *Scedosporium* species (22% for *S. prolificans* and 81% for *S. apiospermum*; *P* < 0.04). None of the formulations adversely affected the PMN antifungal activity. These findings may be important in designing better strategies for management of infections due to these organisms.

Keywords: amphotericin B formulations, *Scedosporium*, polymorphonuclear leucocytes

Introduction

*Scedosporium prolificans* and *Scedosporium apiospermum* (*Pseudallescheria boydii*) are emerging fungal pathogens that cause fatal disseminated infections in immunocompromised hosts and localized infections in immunocompetent patients.¹,² Clinical isolates possess *in vitro* resistance to flucytosine and amphotericin B (AMB), as well as variable susceptibility to itraconazole and the newer azoles voriconazole and posaconazole.¹,³

Deoxycholate AMB (AMBDC), a polyene that interacts with the fungal cell membrane, causing cell death, is often considered the ‘gold standard’ of antifungal agents. However, its administration is frequently complicated by dose-limiting nephrotoxicity. AMB lipid complex (ABLC) and liposomal AMB (LAMB) are lipid formulations developed to reduce these nephrotoxic complications. Both formulations have fewer adverse reactions than conventional AMBDC.

The main host defences against filamentous fungi consist of tissue and circulating phagocytes. Polymorphonuclear leucocytes (PMNs) are the main mediators of host defence against hyphae of filamentous fungi.⁴ Hyphae of *S. prolificans* are damaged by PMNs in a dose-responsive manner.⁵ AMBDC has variable effects on phagocytes; these have been studied by several investigators,⁶–⁸ including the stimulatory effects of AMBDC at clinically relevant concentrations on macrophages and immunostimulatory effects in mice.⁵

Improvement of antifungal therapy against *Scedosporium* spp. is urgently needed. Whether the combination of AMB lipid formulations enhances the antifungal effect of PMNs against these emerging pathogens is not known. We therefore investigated the antifungal effects of human PMNs and AMB

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lipid formulations alone and in combination against *S. prolificans* and *S. apiospermum*.

**Materials and methods**

Heparinized whole blood was obtained from healthy adult volunteers. PMNs were separated by dextran sedimentation and centrifugation over Ficoll.\(^5\) Pooled serum was collected from healthy donors and stored at −35°C for <1 month.

The isolate of *S. prolificans* CM906 used for these studies is the type strain CBS 465.74, stored in the Instituto de Salud Carlos III, Madrid, Spain (kindly donated by Dr Juan Luis Rodriguez-Tudela), which was originally isolated from a case of osteomyelitis. *S. apiospermum* SA1216 was isolated from a biopsy of an infection of the leg. Both isolates caused fatal infections in experimental animals. The inocula were prepared as described previously.\(^5\)

AMBDC (Bristol-Myers Squibb, La Grande Nord, Paris, France), ABLC (Liposome Company, Inc., Princeton, NJ, USA) and LAMB (Gilead Sciences, San Dimas, CA, USA) were used. For *S. prolificans*, the final concentrations of AMBDC, ABLC and LAMB were 0.625, 0.125 and 0.625 µg/mL, and for *S. apiospermum* they were 0.312, 0.062 and 0.625 µg/mL, respectively. These concentrations were selected as the most appropriate for use from separate dose–response XTT experiments. In these experiments, different drug concentrations were mixed with hyphae of the two *Scedosporium* spp. and incubated according to the method described below. The drug concentrations chosen to be used in combination with PMNs were those that achieved <50% activity against the hyphae alone.

The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium-5-carboxanilide sodium salt (XTT dye; Sigma, St Louis, MO, USA) plus coenzyme Q (2,3-dimethoxy-5-methyl-1,4-benzoxquinone; Sigma) assay was used as described previously.\(^3\) A suspension containing 7.5 × 10⁴ conidia/mL in 200 µL yeast nitrogen base supplemented with 2% glucose (YNB) was plated in each well of a 96 flat-bottomed well cell culture cluster (Corning Inc., New York, NY, USA) and incubated for 18 h at 32°C. YNB was then replaced by RPMI 1640 supplemented with 10% pooled human serum; PMNs were added to appropriate wells at an effector/target ratio of 5:1, and antifungal agents were added at specified concentrations. After incubation at 37°C with 5% CO₂ for 3 h, PMNs were lysed by washing three times with H₂O and shaking for 5 min at room temperature before adding 150 µL of PBS containing 0.25 mg/mL XTT plus 40 µg/mL coenzyme Q. After incubation at 37°C with 5% CO₂ for 1 h, the wells were aspirated dry and 100 µL aliquots were transferred and read in a spectrophotometer at 450 nm. Antifungal activity was calculated as percentage hyphal damage = \((1 − \frac{X}{C}) \times 100\), where X is the optical density of test wells and C is the optical density of control wells with hyphae only.

Each experiment was performed with cells of one donor and by use of duplicate or quadruplicate wells for each condition. The average value of these replicate wells was taken as the value for this particular donor/experiment. The averages of each experiment were then used to calculate the mean ± standard error of mean (S.E.M.). Differences between mean values were statistically evaluated by Wilcoxon or Mann–Whitney non-parametric test, as appropriate. A P value of <0.05 indicated significance.

**Results**

The effects of AMBDC in combination with PMNs on hyphae of *S. prolificans* were studied initially (Figure 1a, left). The hyphal damage produced by the combination of the drug with PMNs was significantly greater than that produced by the drug alone (mean ± S.E.M. of hyphal damage produced by the combination was 52 ± 3.9% as compared with 22.9 ± 6.6% for AMBDC alone; *P* = 0.029). However, AMBDC did not have any effect on PMN antifungal function expressed as hyphal damage, since the combination of drug with PMNs exhibited equivalent hyphal damage to the PMNs alone.

When AMBDC was added to *S. apiospermum* (Figure 1a, right), damage to hyphae was greater with the combination of drug and PMNs than with the drug alone (45 ± 8.9% as compared with 7.2 ± 10.4%), but this difference was not significant. Similarly, the antifungal activity of the combination was not significantly different from that of PMNs alone.

**Figure 1.** (a) Percentage of hyphal damage produced by AMBDC alone, human PMNs alone or the combination of AMBDC and PMNs against *S. prolificans* (SP) and *S. apiospermum* (SA). (b) Percentage of hyphal damage produced by ABLC alone, PMNs alone or the combination of ABLC and PMNs against SP and SA. (c) Percentage of hyphal damage produced by LAMB alone, PMNs alone or the combination of LAMB and PMNs against SP and SA. Columns represent the mean ± S.E.M. hyphal damage of four experiments with AMBDC alone, six experiments with ABLC alone, eight experiments with LAMB alone (light grey columns), PMNs alone (white columns) or AMBDC, ABLC or LAMB in combination with PMNs (dark grey columns). An asterisk indicates statistically significant difference from the combined treatment of AMBDC (a), ABLC (b) or LAMB (c) with PMNs for each mould.
The effect of ABLC in combination with PMNs on both organisms was also studied (Figure 1b). The antifungal activity of the combination of ABLC and PMNs against \textit{S. prolificans} and \textit{S. apiospermum} was significantly greater as compared with PMNs or drug alone. For example, while PMNs damaged 46.4 \pm 4.5\% of \textit{S. prolificans} hyphae, the addition of ABLC with PMNs damaged 56.8 \pm 4.9\%, achieving an enhancement of 22\% (\textit{P} = 0.031). Similarly, while LAMB alone damaged 37.4 \pm 8\% of \textit{S. apiospermum} hyphae, the combination of ABLC and PMNs damaged 68 \pm 3.6\%, achieving an enhancement of 81\% (\textit{P} = 0.013).

The hyphal damage produced by the combination of LAMB and PMNs was also greater than that produced by the drug alone (Figure 1c). Hyphal damage of \textit{S. prolificans} was 46.7 \pm 6.1\% for the combination compared with 13.8 \pm 5.7\% for LAMB alone (\textit{P} < 0.001). \textit{S. apiospermum} hyphal damage was 39.3 \pm 8.9\% with the combination compared with 23.1 \pm 9.4\% for LAMB alone; however, this difference did not reach significance. The effect of the combination of LAMB and PMNs was similar to that of PMNs alone on both \textit{S. prolificans} and \textit{S. apiospermum} hyphae.

**Discussion**

It has recently been reported that voriconazole, a newly developed azole, has an additive effect with human PMNs on \textit{Aspergillus fumigatus} hyphal damage.\textsuperscript{9} To our knowledge, despite the wide clinical use of AMB, this is the first time that the effects of lipid formulations of AMB have been studied in combination with phagocytes on hyphal damage in filamentous fungi. Concentrations of AMB formulations that are most appropriate to assess their effect when combined with PMNs have been chosen for these studies.

AMB forms barrel-like pores, which allow ions to traverse the lipid bilayer. This results in depletion of ion gradients leading to electrolyte leakage and cell death. Other proposed mechanisms include lipid peroxidation, inhibition of membrane enzymes, blockade of endocytosis and immune stimulation.\textsuperscript{8} In the case of ABLC, AMB is concealed within ribbon-like structures. The release of lipases by the fungus breaks down the ribbon-like structures, thus releasing AMB and increasing its local concentration. This could enhance local antifungal activity of AMB and increase the permeability of the fungal membrane to PMN fungicidal products. If \textit{Scedosporium} spp. release lipases, an enhanced delivery of drug and PMN fungicidal products to the hyphal membrane could ensue, which might account for the additive effect observed.

The complex ribbons may also exert a direct effect on PMNs (e.g. activating them further or increasing their antifungal functions) or on hyphae, rendering them more susceptible to PMNs. AMB has been found to increase the killing of phagocytosed \textit{A. fumigatus} conidia and \textit{Candida albicans} blastoconidia by phagocytes. In addition, it enhances their adherence\textsuperscript{6,7,10} and reduces cell viability. The effects of AMB on superoxide anion production are variable; some authors have found priming or enhancement,\textsuperscript{7} whereas others have found an inhibitory effect\textsuperscript{8} or no effect.\textsuperscript{10}

Little is known about the effects of ABLC or LAMB on superoxide anion production by PMNs. As a first attempt to address the mechanism of this combination result, the effect of the three AMB formulations on the oxidative burst of PMNs was studied in our laboratory. None of these formulations affected the superoxide anion produced by PMNs, either unstimulated or stimulated by formylmethionyl-leucylphenylalanine (data not shown). Regardless of the mechanism(s) of these additive effects, the findings of additive antifungal action of ABLC and PMNs described here suggest that interactions between phagocytes and pharmaceutical compounds merit exploration against other filamentous fungi that cause life-threatening infections.

Although granulocyte transfusions are controversial, encouraging reports of efficacy of transfusions of granulocyte-colony stimulating factor-mobilized granulocytes in patients with mycoses refractory to antifungal chemotherapy\textsuperscript{11} lend support to this strategy in \textit{Scedosporium} spp. infection. Improved PMN function through withdrawal of immunosuppressants or through enhancement by recombinant cytokines may optimize the management of non-neutropenic patients with infection due to these emerging pathogens.

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**References**


