Liposomal Amphotericin B Displays Rapid Dose-Dependent Activity against *Candida albicans* Biofilms

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Biofilms formed by *Candida albicans* bloodstream isolates on catheters are an important clinical problem. Devising chemotherapeutic strategies to treat these *in situ* is an attractive option. We report here that liposomal amphotericin effectively kills *C. albicans* biofilms rapidly (12 h) and effectively (>90%) in a dose-dependent manner, whereas caspofungin displays an inverse concentration-dependent effect. This study has implications for considering the effective doses of antifungal agents used for catheter lock therapy.

While there is no question that the use of various medical devices has greatly facilitated the management of serious medical and surgical conditions, the introduction of artificial materials into various anatomical locations has been accompanied by the ability of *Candida albicans* to colonize and form biofilms on devices such as shunts, stents, endotracheal tubes and various types of catheters (1). In fact, it has been reported that biofilm-forming *C. albicans* bloodstream isolates were significantly correlated with increased mortality (2).

Bloodstream infections due to *C. albicans* remain an important cause of morbidity and mortality worldwide. It was reported that at least 1 in 4 hospitalized patients who contract a candidemia die before discharge (3). The intensive care unit (ICU) is of pivotal importance in terms of developing a candidemia, where the use of central venous catheters is extremely common and associated with candidal sepsis (4). It was reported that 45.4% cases of candidemia were associated with the ICU, with an overall 30-day mortality of 26.4%. Removal of the central venous catheter was associated with a significant reduction in mortality (5). There are currently no guidelines for treating *C. albicans*-associated biomaterial infections with chemotherapeutic agents, other than physical removal of the catheter (6). However, limited anecdotal evidence exists for *in situ* use of antifungal lock therapy (ALT); nevertheless, the use of amphotericin B deoxycholate to resolve a catheter-related infection has been reported to be successful (7–9).

This study aims to investigate and compare the use of key antifungal agents classes (azole, polyene, and echinocandin) against a range of *C. albicans* bloodstream isolates growing as biofilms, with the objective of advocating their use in ALT. One hundred *C. albicans* bloodstream strains obtained from a Scottish candidemia study were selected for testing (10). All isolates were maintained on Sabouraud agar (SAB) at 30°C and propagated in yeast-peptone-dextrose (Sigma, Poole, United Kingdom) medium in an orbital shaker (100 rpm) at 30°C overnight. Cells were harvested, washed in sterile phosphate-buffered saline (PBS; Sigma, Poole, United Kingdom), then resuspended in RPMI 1640 buffered with morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich, Dorset, United Kingdom), counted, and standardized using an improved Neuber hemocytometer.

Initially, antifungal testing to determine planktonic MICs (PMICs) was performed using the CLSI M-27A broth microdilution methodology (11). The following antifungal agents, prepared in double-distilled water (ddH_2O), were used in the course of this study against 100 *C. albicans* isolates: liposomal amphotericin B (AMB) (AmBisome; Gilead Sciences, Cambridge, United Kingdom), caspofungin (CSP) (Cancidas; Merck Sharp & Dohme, Hertfordshire, United Kingdom), and voriconazole (VRZ) (Vfend; Pfizer Pharmaceuticals). AMB, CSP, and VRZ were highly effective against these isolates, exhibiting $PMIC_{50}$ values of 0.125, 0.0625, and 0.125 mg/liter, respectively (Table 1). The $PMIC_{90}$ for all three antifungal agents was 0.125 mg/liter for all 100 isolates. These data are in agreement with previous literature (12).

Next, sessile susceptibility testing was performed as described previously (13). Biofilms were formed using standardized cell suspensions (200 μl of 1×10^6 cells/ml), added to selected microtiter wells, and incubated for 48 h at 37°C. After washing the biofilms, each antifungal agent was then added in serially double-diluted concentrations and incubated for a further 24 h at 37°C. A semiquantitative measure of biofilm killing was assessed using a formazan salt-based XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide) reduction assay (13). Sessile MICs (SMICs) were determined at a ≥80% XTT reduction. Testing of these isolates was performed in triplicate. All *C. albicans* isolates formed robust biofilms, as assessed by XTT and crystal violet (data not shown). AMB exhibited good overall activity, with $SMIC_{50/90}$ of 4 and 16 mg/liter, respectively, ranging from 2 to 64 mg/liter. For CSP, the $SMIC_{50/90}$ was 0.25 mg/liter and 1 mg/liter, respectively, ranging from ≤0.0625 to 8 mg/liter. VRZ sessile activity was highly ineffective, with no notable activity against any strain tested ($SMIC_{50/90}$ of ≥64 mg/liter).

We next undertook a comparative time-kill evaluation of *C. albicans* biofilms ($n = 10$) (Fig. 1). These isolates exhibiting good biofilm formation were selected based on high biomass and met-

TABLE 1 Planktonic and sessile sensitivities of *Candida albicans* biofilms treated with three antifungal classes

Parameter	MIC value (mg/liter) with[a]:								
	VRZ			AMB			CSP		
	PMIC	SMFC 50%	SMFC 80%	PMIC	SMFC 50%	SMFC 80%	PMIC	SMFC 50%	SMFC 80%
MIC range	≤0.0625–2	>64	>64	≤0.0625–0.25	<0.5–32	2–64	0.0625–0.25	≤0.0625–0.5	≤0.0625–8
MIC_{50}	0.125	≥64	>64	0.0625	0.5	4	0.0625	≤0.0625	0.25
MIC_{90}	0.125	>64	>64	0.125	2	16	0.125	0.5	1

[a] SMFC 50% and SMFC 80%, sessile minimum fungicidal concentration at a 50% and 80% reduction of XTT metabolism, respectively, in comparison to an untreated control.

abolic activity. Biofilms were washed prior to addition of AMB, CSP, and VRZ at concentrations of 1×, 2×, 4×, and 8× the MIC_{50}. Biofilms were incubated in the presence of each antifungal compound for 2, 4, 8, 12, 24, 48, and 72 h, and their metabolic activities were assessed using an XTT reduction assay. Untreated

FIG 1 Time-kill kinetics of *Candida albicans* biofilms challenged with liposomal amphotericin B (A), caspofungin (B), and voriconazole (C) at 1×, 2×, 4×, and 8× $SMIC_{50}$ and measured by the XTT reduction assay. Data points represent 10 individual clinical isolates in replicate ($n = 10$). This was analyzed by a two-way ANOVA, with error bars representing the standard deviations.

biofilms containing RPMI 1640 served as appropriate comparative controls for each isolate at each time point. Ten replicate biofilms were included for each condition tested, with testing performed on two separate occasions. A two-way analysis of variance (ANOVA) was performed on transformed data using SPSS Software (Chicago, IL) and a Bonferroni posttest to allow different drug concentrations or different time points to be compared. AMB challenge was shown to be rapid and dose-dependent for all concentrations tested, with a >90% kill observed for all concentrations after 12 h (Fig. 1A). Significant differences were observed between each concentration, except between 2× and 4× $SMIC_{50}$, at each time point up until 12 h ($P < 0.0001$). No significant differences were observed thereafter. At 8× $SMIC_{50}$, the activity was significantly more rapid after 4 and 8 h ($P < 0.0001$), reducing metabolism by 60% and 94%, respectively. CSP challenge showed significant differences between concentrations tested in a time-dependent manner ($P < 0.0001$), but with an inverse relationship between time-kill characteristics and the concentration tested, i.e., the most effective and rapid concentration of CSP was 1× $SMIC_{50}$, resulting in a 99% kill after 24 h (Fig. 1B). This was followed by 2×, 4×, and 8× $SMIC_{50}$, which caused 98, 90, and 85% reductions after 24 h, respectively. Both 1× and 2× $SMIC_{50}$ were significantly superior compared to 4× and 8× $SMIC_{50}$ from 12 h onwards ($P < 0.0001$). VRZ time-kill studies showed dose-dependent characteristics, but with only an 18% kill after 72 h at 8× MIC_{50} (Fig. 1C). No significant differences were observed between any of the concentrations tested ($P > 0.05$).

Collectively, these data demonstrate that both AMB and CSP have the potential for direct *in situ* treatment of *C. albicans* biofilms on infected catheters, indwelling biomaterials, or tissue. While AMB did not achieve a 99% kill like that of CSP after 24 h, its kill was rapidly effective (~95%) after only 12 h of treatment. Moreover, at 1× MIC_{50}, the activity was not significantly different from that of the other concentrations tested at this time, suggesting saturation of the drug within the biofilm. This may be because of the liposomal formulation of AMB that permits greater diffusion through the biofilm. It has been shown that liposomal formulations have the potential for treatment of catheters *in vivo* (14). *In vitro* investigations have also reported the potential benefit of this approach, either as a single agent or in combination with EDTA (15–17), though the range of concentrations tested is highly variable. In contrast, CSP showed paradoxical activity against the *C. albicans* biofilms (18). Recent literature suggests adaptive resistance to echinocandins through cell wall remodelling, specifically increased chitin content (19). It is plausible that higher concentrations of CSP induce these adaptive changes, accounting for this effect.

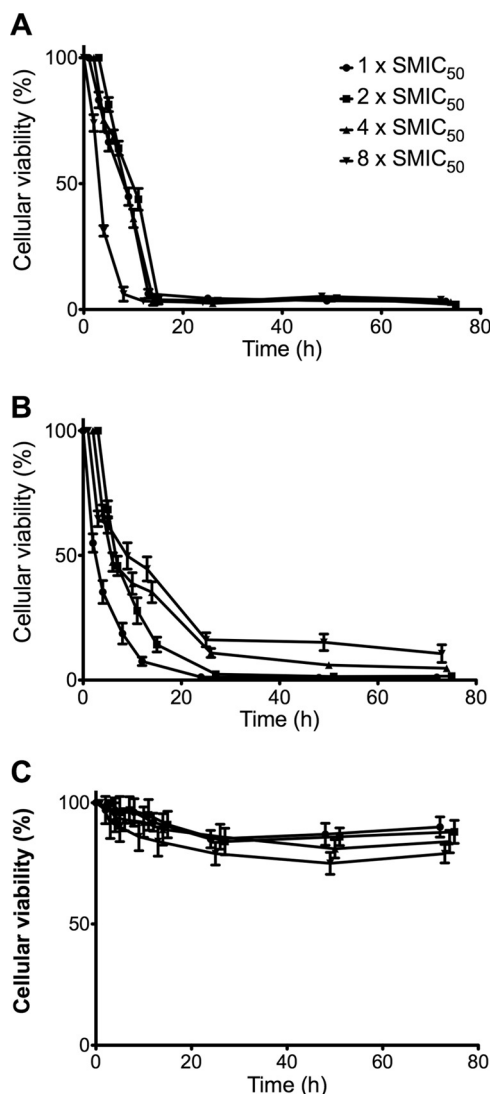Recent clinical data have shown that patients with defined bio-

film-forming bloodstream *C. albicans* isolates experienced a short length of hospital stay and low mortality rates when treated with echinocandins and liposomal amphotericin B (20). These observations generally support our findings, yet clinically not all patients respond to antifungal treatment equally. This may be partly because of the level of biofilm formation by individual strains and/or differential biofilm response to different antifungal classes. Interestingly, none of the antifungal agents tested killed the biofilms in their entirety, suggesting adjunctive therapy is required (15). Further studies are required to understand how to maximize antifungal activity against *C. albicans* biofilms and improve their clinical management.

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