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In vitro activity of antifungal combinations against *Candida albicans* biofilms

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Objectives: The aim of the present study was to evaluate the *in vitro* activity and synergism of the combinations of amphotericin B/caspofungin and amphotericin B/posaconazole against *Candida albicans*, grown either as planktonic cells or in biofilms.

Methods: Ten *C. albicans* bloodstream isolates used in this study were collected from intensive care patients admitted to the Vienna University Hospital between 2006 and 2007. Chequerboard tests were employed to determine the efficacy of the antifungal combinations amphotericin B/caspofungin and amphotericin B/posaconazole against both planktonic cells and biofilms. *C. albicans* biofilms were prepared using the static microtitre plate model. The activity of antifungal combination therapy was determined by visual reading for planktonic cells and using the XTT assay for biofilms.

Results: For *Candida* biofilms the median MIC was 4 mg/L for amphotericin B and caspofungin, and >256 mg/L for posaconazole. The combination amphotericin B/posaconazole yielded synergism [fractional inhibitory concentration index (FICI) <0.26], whereas amphotericin B/caspofungin yielded indifferent interaction only (FICI 0.75–1.25) against all isolates when grown in biofilms. Under planktonic conditions, synergism was demonstrable for the combination amphotericin B/caspofungin against 4 of the 10 isolates, whereas the combination of caspofungin/posaconazole was indifferent against all tested isolates.

Conclusions: We showed that MICs for planktonic and biofilm forms of *C. albicans* were much lower when treated with an antifungal combination than when treated with single agents. The combination of amphotericin B/posaconazole yielded synergism against *Candida* biofilms, whereas amphotericin B/caspofungin yielded indifferent interaction.

Keywords: *C. albicans*, amphotericin B, caspofungin, posaconazole

Introduction

Invasive *Candida* infections are associated with high morbidity and mortality in immunocompromised and severely ill patients.[1] *Candida* may colonize surfaces of foreign inserted material, most commonly vascular catheters, but also surfaces of implants, such as artificial valves or hearts, forming persistent biofilms. Whereas percutaneous vascular catheters may be removed quickly, the removal of implanted medical devices is problematic because these implants generally have a life-supportive function. Thus, any efforts towards successful treatment and retaining the implanted device are urgently needed in clinical practice. Newly developed antifungal agents such as caspofungin and posaconazole show excellent *in vitro* activity against *Candida* planktonic cells.[2] Echinocandins, including caspofungin as the first echinocandin introduced for clinical use, were shown to be more active against *Candida albicans* biofilm than the azoles.[3,4] To improve the activity against *Candida* biofilms and *Candida* biofilm-associated infections, the use of the new antifungals in combination might be more successful.

The aim of the present study was to evaluate the *in vitro* activity and synergism of antifungal combinations including caspofungin, posaconazole and amphotericin B against *C. albicans* in both growth forms, planktonic cells and biofilms.

Materials and methods

Fungal isolates

Ten *C. albicans* bloodstream isolates used in this study were collected from intensive care patients admitted to the Vienna University Hospital between 2006 and 2007. All isolates were identified using standard

procedures and stored at −70°C. *C. albicans* ATCC 10231 was used as a quality control strain.

Antifungal drugs

A standard antifungal powder of amphotericin B was purchased from the manufacturer (Bristol-Myers Squibb, Epernon, France), prepared in 100% DMSO at a concentration of 1000 mg/L. Caspofungin and posaconazole were purchased as the products for clinical use (Cancidas®, Merck & Co., Inc., 50 mg of powder for intravenous infusion; Noxafil®, Schering-Plough Co., 40 mg/mL oral suspension).[5] The Cancidas® powder was diluted in distilled water to 1000 mg/L. All antifungal drugs were diluted in RPMI 1640 broth with L-glutamine and without sodium bicarbonate, buffered with MOPS to final concentrations of 0.06–16 mg/L for amphotericin B and caspofungin, and 0.06–256 mg/L for posaconazole.

In vitro activity of antifungal combinations against planktonic cells of C. albicans

The individual MICs for planktonic cells were determined using the microdilution method in accordance with the guidelines of the CLSI (formerly the NCCLS).[6] Chequerboard tests were employed to determine the efficacy of antifungal combinations.[7] The drugs used in combination were amphotericin B/caspofungin and amphotericin B/posaconazole at the concentrations described above.

MICs of individual drugs determined by visual readings correspond to either a complete (100% for amphotericin B and caspofungin) or a prominent (50% for posaconazole) decrease in turbidity compared with the growth control. MICs of drug combinations correspond to complete growth inhibition for the amphotericin B/caspofungin combination and prominent growth inhibition for the amphotericin B/posaconazole combination.

In vitro activity of antifungal combinations against biofilms of C. albicans

The effects of amphotericin B, caspofungin and posaconazole alone, and antifungal combinations of amphotericin B/caspofungin and amphotericin B/posaconazole, on *C. albicans* biofilms were assessed by a chequerboard microdilution method with biofilms formed in the wells of microtitre plates and an XTT-based colorimetric assay.[8] The MIC for biofilms was determined as compared with control prominent increase in optical density (OD) (by 50%) of wells containing biofilms and antifungal drugs alone or in combination.

Interpretation of drug combination interaction

Drug combination interaction was classified on the basis of the fractional inhibitory concentration index (FICI).[7] The FICI was calculated by the formula: $FICI = (A_c/A_a) + (B_c/B_a)$, where A_c and B_c are the MICs of antifungal drugs in combination, and A_a and B_a are the MICs of antifungal drugs A and B alone. The interaction was defined as synergistic if the FICI was ≤0.5, indifferent if the FICI was >0.5 and ≤4, and antagonistic if the FICI was >4.0.

Growth inhibition assay on C. albicans biofilm

To test the fungicidal activity of antifungal drugs alone or in combination against biofilms, biofilms were prepared and treated with antifungal agents as described above. After incubation with antifungal drugs, biofilms were scraped off and seeded on Sabouraud agar plates. Following incubation at 35°C for 48 h, the number of cfu on each plate was determined.

Statistical analysis

Each experiment was performed in duplicate, and repeated at least three times on different days. For the growth inhibition assay the arithmetic mean and standard error obtained on three different occasions were calculated. Significance of difference ($P < 0.05$) was assessed using the Mann–Whitney *U*-test.

Results

MICs for C. albicans under planktonic conditions

The MIC of individual antifungal drugs for planktonic cells of 10 clinical *C. albicans* isolates ranged from 0.25 to 0.5 mg/L for amphotericin B, from 0.25 to 1 mg/L for caspofungin, and from 0.06 to 0.125 mg/L for posaconazole. The MIC for *C. albicans* ATCC 10231 was 0.25 mg/L for amphotericin B and caspofungin and 0.06 mg/L for posaconazole. The MICs of the combinations amphotericin/caspofungin and amphotericin B/posaconazole, as well as the MICs of the single drugs tested, are given in Table 1. The FICI values ranged from 0.37 to 0.74 for the combination of amphotericin B/caspofungin, and from 0.6 to 2.0 for amphotericin B/posaconazole. For the combination amphotericin B/caspofungin, synergism was achieved in 4 of the 10 *C. albicans* isolates. The combination amphotericin B/posaconazole yielded indifferent interaction against all 10 *C. albicans* isolates.

MICs for C. albicans biofilms

The median MIC of individual drugs for *C. albicans* biofilms was 4 mg/L for amphotericin B, 4 mg/L for caspofungin and >256 mg/L for posaconazole. The MIC of amphotericin B, in combination with either posaconazole or caspofungin, decreased 2- to 8-fold for *C. albicans*. The MIC of posaconazole, in combination with amphotericin B, decreased from >256 mg/L to 2–4 mg/L (Table 1). Therefore, the combination of amphotericin B/posaconazole yielded synergism against the biofilms of all 10 *C. albicans* isolates (FICI < 0.27). The combination of amphotericin B/caspofungin yielded an indifferent interaction against all 10 *C. albicans* isolates (FICI 0.75–1.25).

Fungicidal activity against Candida biofilms

Figure 1 shows the fungicidal activities of antifungal drugs alone and in combination. Against biofilms, amphotericin B and caspofungin at concentrations of 1 and 2 mg/L and posaconazole at concentrations of 2 and 256 mg/L failed to reduce the fungal colony counts significantly compared with the untreated control (<1 $\log_{10}$ cfu/mL; $P > 0.05$). The combination of 1 mg/L amphotericin B/2 mg/L caspofungin significantly reduced the growth of the cells in biofilm by >1 $\log_{10}$ cfu/mL ($P < 0.05$). The greatest decrease in the growth of the cells within the biofilm, reaching >2 $\log_{10}$ cfu/mL ($P < 0.05$), was achieved by the combination of 1 mg/L amphotericin B and 2 mg/L posaconazole.

Table 1. MICs of amphotericin B (AMB), caspofungin (CAS) and posaconazole (POS), alone and in combination, and fractional inhibitory concentration index (FICI) results against planktonic and biofilm forms of 10 clinical *C. albicans* bloodstream isolates and quality control strain ATCC 10231

	Planktonic cells					Biofilm				
	MIC (mg/L)			MIC [drug A+drug B (mg/L)] (FICI)		MIC (mg/L)			MIC [drug A+drug B (mg/L)] (FICI)	
C. albicans	AMB	CAS	POS	AMB+CAS	AMB+POS	AMB	CAS	POS	AMB+CAS	AMB+POS
ATCC 10231	0.25	0.25	0.06	0.06+0.125 (0.74)	0.06+0.06 (1.24)	4	4	>256	1+2 (0.75)	1+2 (<0.26)
7185	0.5	1	0.125	0.25+0.06 (0.56)	0.06+0.125 (1.12)	4	2	>256	1+1 (0.75)	1+2 (<0.26)
1240	0.25	1	0.125	0.125+0.06 (0.56)	0.125+0.125 (1.5)	4	2	>256	1+1 (0.75)	1+2 (<0.26)
16406	0.5	1	0.06	0.125+0.125 (0.38)	0.06+0.06 (1.12)	4	2	>256	1+2 (1.25)	1+2 (<0.26)
6039	0.5	0.5	0.125	0.125+0.06 (0.37)	0.06+0.06 (0.6)	2	4	>256	1+1 (0.75)	0.5+2 (<0.26)
14291	0.25	0.25	0.06	0.06+0.06 (0.48)	0.06+0.06 (1.24)	4	2	>256	1+1 (0.75)	0.5+2 (<0.13)
7582	0.25	0.5	0.06	0.125+0.06 (0.62)	0.25+0.06 (2.0)	2	4	>256	0.5+2 (0.75)	0.5+2 (<0.26)
3390	0.25	0.5	0.125	0.125+0.06 (0.62)	0.125+0.06 (0.98)	2	4	>256	0.5+2 (0.75)	0.5+4 (<0.27)
5443	0.5	0.5	0.125	0.25+0.06 (0.62)	0.25+0.06 (0.98)	2	4	>256	1+2 (1.0)	0.5+4 (<0.27)
1128	0.5	0.5	0.125	0.125+0.06 (0.37)	0.125+0.06 (0.73)	4	4	>256	1+2 (0.75)	1+4 (<0.27)
5956	0.5	0.5	0.06	0.25+0.06 (0.62)	0.125+0.06 (1.12)	4	4	>256	1+2 (0.75)	0.5+4 (<0.14)

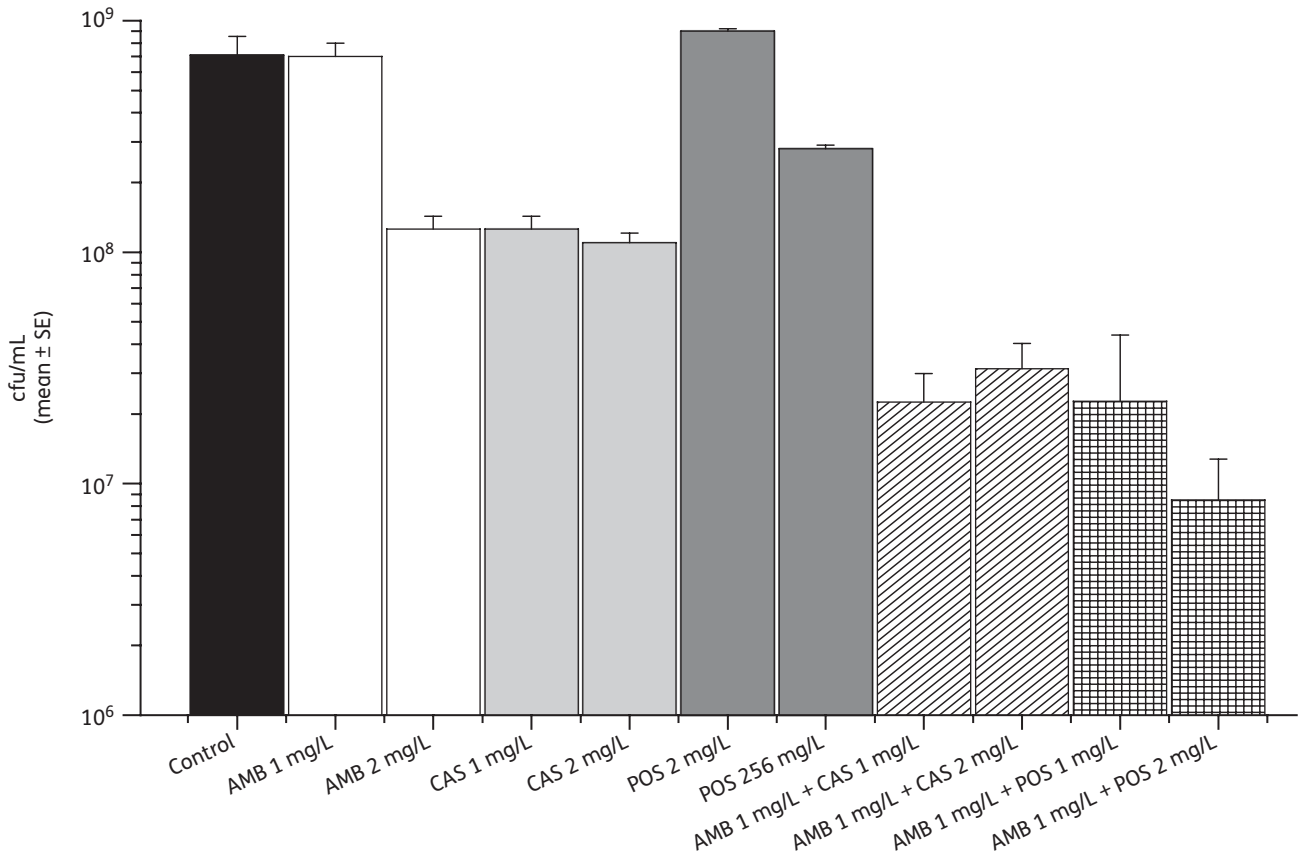

Figure 1. Reduction of the fungal colony counts (cfu/mL) in the biofilms of 10 invasive *C. albicans* isolates after incubation with amphotericin B (AMB), caspofungin (CAS) and posaconazole (POS) alone or in combination. The concentrations of antifungal drugs alone or in combination are in accordance with the MICs determined using the XTT assay.

Discussion

In the present work, a chequerboard assay and fungicidal assay were used to evaluate the interaction of amphotericin B with either caspofungin or posaconazole against planktonic cells and biofilms of *C. albicans in vitro*. As reported in previous studies,[2] all tested antifungal agents were highly active against the planktonic cells of *C. albicans*. However, significant increases in the MICs of all antifungals tested were observed for *C. albicans* biofilms.[4,5]

The use of antifungal combinations may improve the management of *Candida* biofilm-associated infection, disrupt the biofilms and prevent the emergence of resistance. Bachmann *et al.*[9] described indifferent interaction with some trend towards additivity of the combination of amphotericin B and caspofungin against a single *C. albicans* isolate *in vitro*. However, there exist no data about the interaction of combinations of amphotericin B and the new triazole agent posaconazole against clinical *Candida* isolates causing invasive infections.

To our knowledge, the *in vitro* phenomenon of synergism of amphotericin B and posaconazole against *C. albicans* biofilms is reported here for the first time. Synergism against *Candida* biofilms was achieved due to reduction of posaconazole resistance expressed by a significant decrease in the MIC of posaconazole when used in combination with amphotericin B. There are many mechanisms for resistance of microbial cells within biofilms, although they are variable and differential. For bacteria, decreased penetration, changes in microbial metabolism and activity, and expression of resistance genes have been discussed. Some of these mechanisms may play a role in fungal biofilms, such as expression of efflux pumps involved in fluconazole resistance.[3] However, little is known about the resistance of *Candida* biofilms to posaconazole. Posaconazole has a unique hydrophobic structure. Biofilms are composed of a hydrophilic matrix, cells and hyphae. Hypothetically, posaconazole used together with amphotericin B may undergo some structural change affecting the biofilm matrix and the fungal cells within the biofilm. In our experiments, the effects of the antifungal combination were determined after a single incubation for 48 h only, even then leading to a significant log reduction of candidal growth.

In clinical practice, simultaneous application of two antifungals is not yet recommended for the treatment of invasive candidiasis, except for the combination of amphotericin B with flucytosine in endocarditis based on expert opinion.[10] Yet there may be circumstances where antifungal combination therapy could be of value. Persistent candidaemia originating from a biofilm-associated infection, such as endocarditis, might justify the deployment of an antifungal combination therapy to control the infection until the patient is stable enough for surgery or transplantation.

In conclusion, synergism and antagonism are *in vitro* concepts that are difficult to translate into clinical practice. Although there are experimental data on combination therapy, clinical studies, which could support the advantage of combination therapy over antifungal monotherapy in biofilm-associated infections, are needed but are hard to perform. Nevertheless, evidence of synergism of antifungal combination therapy *in vitro* might be the first step in establishing appropriate antifungal therapy.

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Transparency declarations

None to declare.

References

1 Eggimann P, Garbino J, Pittet D. Management of *Candida* species infections in critically ill patients. *Lancet Infect Dis* 2003; **3**: 772–85.

2 Espinel-Ingroff A, Pfaller M, Messer SA *et al*. Multicenter comparison of the Sensititre YeastOne Colorimetric Antifungal panel with the NCCLS M27-A2 reference method for testing new antifungal agents against clinical isolates of *Candida* spp. *J Clin Microbiol* 2004; **42**: 718–21.

3 Mukherjee PK, Chandra J, Kuhn DM *et al*. Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect Immun* 2003; **71**: 4333–40.

4 Kuhn DM, George T, Chandra J *et al*. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob Agents Chemother* 2002; **46**: 1773–80.

5 Tobudic S, Lassnigg A, Kratzer C *et al*. Antifungal activity of amphotericin B, caspofungin and posaconazole on *Candida albicans* biofilms in intermediate and mature development phases. *Mycoses* 2009; doi:10.1111/j.1439-0507.2009.01690.x.

6 National Committee for Clinical Laboratory Standards. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts-Second Edition: Approved Standard M27-A2*. NCCLS, Wayne, PA, USA, 2002.

7 Moody JA. Synergism testing. Broth microdilution checkerboard and broth microdilution methods, In: Isenberg HD, ed. *Clinical Microbiology Procedures Handbook, Volume 1*. Washington, DC: American Society for Microbiology, 1991; 5.18.1–28.

8 Ramage G, Vande WK, Wickes BL *et al*. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* 2001; **45**: 2475–9.

9 Bachmann SP, Ramage G, VandeWalle K *et al*. Antifungal combinations against *Candida albicans* biofilms in vitro. *Antimicrob Agents Chemother* 2003; **47**: 3657–9.

10 Pappas PG, Kauffman CA, Andes D *et al*. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* 2009; **48**: 503–35.