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Susceptibility of *Candida albicans* biofilms to azithromycin, tigecycline and vancomycin and the interaction between tigecycline and antifungals

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ABSTRACT

Despite growing data on antimicrobial lock therapy (ALT) in treating bacterial catheter-related bloodstream infections (CR-BSIs), ALT has not been established as a treatment option for CR-BSI caused by *Candida albicans*. Based on our finding that high-dose doxycycline exhibited antifungal activity against mature *C. albicans* biofilms, we evaluated additional antibacterial agents with Gram-positive activity [azithromycin, tigecycline (TIG) and vancomycin]. After screening these antibiotics, it was found that TIG had substantial antifungal activity against mature *C. albicans* biofilms. Therefore, TIG was assayed alone and in combination with fluconazole (FLC), amphotericin B (AmB) or caspofungin (CAS). TIG at 2048 μg/mL resulted in a >50% reduction in the growth of planktonic *C. albicans* cells. TIG inhibited the formation of biofilms from 128 μg/mL. Against mature biofilms, 2048 μg/mL TIG reduced metabolic activity by 84.2%. Furthermore, addition of 512 μg/mL TIG to FLC at all concentrations tested provided additional reduction in the metabolic activity of mature biofilms. However, this was not superior to 512 μg/mL TIG alone. TIG at 512 μg/mL increased the antifungal effect of lower concentrations of AmB (0.03125–0.25 μg/mL), but at 0.03125 μg/mL and 0.0625 μg/mL this effect was not superior to 512 μg/mL TIG alone. TIG inhibited the antifungal effect of higher concentrations of AmB (≥2 μg/mL). TIG at 512 μg/mL inhibited the antifungal activity of CAS at lower concentrations (0.25–8 μg/mL). These data indicate that high-dose TIG is highly active in vitro against planktonic cells, forming biofilms and mature biofilms of *C. albicans*.

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1. Introduction

Catheter-related bloodstream infections (CR-BSIs) result in significant morbidity and mortality, increased length of stay and increased cost of care in patients requiring intravascular devices. The incidence of CR-BSI has paralleled the increased use of intravascular devices such as central venous catheters and those used in haemodialysis over the past 2 decades [1]. *Candida albicans* has emerged as a significant pathogen causing CR-BSI, especially amongst immunosuppressed and hospitalised patients. Although Gram-positive bacteria such as *Staphylococcus epidermidis* and *Staphylococcus aureus* have been found to be the most common pathogens associated with CR-BSI, epidemiological studies have indicated that *Candida* spp. are the fourth leading cause [1,2].

Biofilm formation by pathogenic microorganisms, including *C. albicans*, plays a key role in infection of intravascular devices and contributes to the difficulty in the management of these device-related infections [3–9]. By the mid 1980s it had been established that bacteria embedded in biofilms (sessile form) were particularly resistant to conventional antibiotics compared with their planktonic (free culture) form [9]. *Candida albicans* biofilms were also found to have greater resistance to the majority of the antifungal agents used in the mid 1990s [10]. Thus, current clinical guidelines recommend removal of these devices as part of the management of CR-BSI caused by *Candida* spp. whenever feasible [11].

Prior to the availability of echinocandins and azoles, the polyene antifungal agent amphotericin B (AmB) was the major antifungal agent used in the management of invasive *Candida* infections. The high incidence of nephrotoxicity of AmB led to the evaluation of combination therapies in the hope of decreasing the amount of AmB required to treat fungal infections thus minimising the risk of nephrotoxicity. These studies performed in the 1970s and 1980s revealed that antibiotics such as rifampicin and tetracyclines enhanced the effect of AmB against various pathogenic yeasts in their planktonic form in vitro [12–17].

Since then, it has been determined that echinocandins and lipid formulations of AmB have activity against *C. albicans* biofilms [18,19]. El-Azizi [20] showed that rifampicin and doxycycline

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(DOX) enhanced the activity of AmB against non-*albicans Candida* spp. biofilms in vitro. Recently, our laboratory has shown that DOX enhances the activity of certain antifungal agents and has intrinsic activity against *C. albicans* biofilms [21].

Thus, we screened several standard Gram-positive antibacterial agents, including azithromycin (AZM), tigecycline (TIG) and vancomycin (VAN), against *C. albicans* biofilms. Of these antibiotics, it was found that TIG had the greatest antifungal activity against mature *C. albicans* biofilms. Therefore, we chose to study TIG in detail and to characterise its activity alone and in combination with AmB, caspofungin (CAS) and fluconazole (FLC) against *C. albicans* biofilms.

2. Materials and methods

2.1. Materials

The clinically derived wild-type SC5314 strain of *C. albicans* was used for this study [22]. AZM and VAN were obtained from Sigma Chemical Co. (St Louis, MO). TIG (Wyeth Pharmaceuticals Inc., Philadelphia, PA) was purchased from the hospital pharmacy (Raymond G. Murphy VA Medical Center, Albuquerque, NM). The antifungal agents FLC and AMB were from Sigma Chemical Co., and CAS (Merck, Whitehouse Station, NJ) was purchased from the hospital pharmacy. Stock solutions of TIG (10 mg/mL) and AZM (100 mg/mL) were prepared in 0.9% (w/v) NaCl and stored at −20 °C and −80 °C, respectively. Stock solutions of AmB (10 mg/mL) were prepared in dimethyl sulphoxide (DMSO) and stored at −80 °C. Sterile water was used to prepare stock solutions of VAN (50 mg/mL), FLC (2 mg/mL) and CAS (7.2 mg/mL). All of the water–soluble agents were stored at −80 °C. All solutions were sterilised by passing through 22 μm filters.

2.2. Effect of tigecycline on the growth of planktonic Candida albicans cells

The effect of TIG on the growth of planktonic *C. albicans* was evaluated by adding an inoculum of yeast cells to complete synthetic media containing TIG at concentrations of 0, 32, 128, 512 and 2048 μg/mL at a starting optical density at 600 nm (OD_{600}) of 0.1. Cells were incubated at 30 °C for 24 h in a shaker at 250 rpm. Aliquots of each culture were collected after 2, 4, 6, 8, 12, 16, 22 and 24 h of incubation. The OD_{600} of the 1:20 dilution of each aliquot was assessed using an Ultrospec™ 2100 pro spectrophotometer (Amersham Biosciences, Piscataway, NJ). Each condition was performed in triplicate. Reductions in the growth curve of treated cells were determined relative to untreated cells.

2.3. Biofilm formation and assessment of metabolic activity

Methods used for *C. albicans* biofilm formation in 96-well polystyrene microtitre plates as well as the XTT reduction assay to determine metabolic activities of the biofilms were performed as described by Ramage and Lopez-Ribot [23]. In brief, *C. albicans* was grown in yeast peptone dextrose broth overnight at 30 °C. Cells were harvested and washed twice with single strength phosphate-buffered saline (1× PBS). Washed cells were then re-suspended in 5 mL of 1× PBS and counted. A final suspension of 10^6 cells/mL was made in RPMI 1640 medium. Then, 100 μL of the cell suspension was added to designated wells of the microtitre plate. To evaluate the effect of TIG on the formation of *C. albicans* biofilms, TIG was added concomitantly to the yeast cell suspension in RPMI 1640 used for the biofilm formation to achieve final concentrations of 1–2048 μg/mL. Cells were then incubated at 37 °C for 24 h to allow the formation of biofilms.

2.4. Antifungal activity against mature biofilms

To evaluate the antifungal activity of antibacterial agents on mature *C. albicans* biofilms, biofilms were formed as described above [23] and were incubated at 37 °C for 24 h. Next, mature biofilms were washed three times with 1× PBS. RPMI 1640 containing doubling concentrations of AZM and VAN (from 2 μg/mL to 2048 μg/mL) and TIG (from 1 μg/mL to 4096 μg/mL) was added to the pre-formed biofilms. Biofilms were then incubated at 37 °C for 24 h. Combination antifungal and TIG experiments were performed by treating mature biofilms with RPMI 1640 containing doubling concentrations of FLC, AmB or CAS, alone and in combination with a fixed concentration of TIG at 512 μg/mL, and incubated for 24 h.

Each biofilm experiment (formation or pre-formed) was performed independently three times in quadruplicate, except for the initial screening evaluation of the Gram-positive antibacterial agents, which was done at least once in triplicate. Drug-free biofilm wells containing only RPMI 1640 were used as controls. Metabolic activities of the biofilms of each set of experiments were assessed using the XTT reduction assay [23]. Production of formazan was measured spectrophotometrically (optical density at 490 nm) on a BioTek ELx808 Microplate Reader (BioTek Instruments Inc., Winooski, VT). Effects of the antimicrobial agents were expressed as a percentage relative to the metabolic activity of the untreated biofilms.

Antifungal activity was defined as a statistically significant reduction in the metabolic activity of mature *C. albicans* biofilms treated by an agent compared with the metabolic activity of untreated biofilms. A paradoxical effect was defined as a statistically significant decrease in antifungal activity by an agent at higher

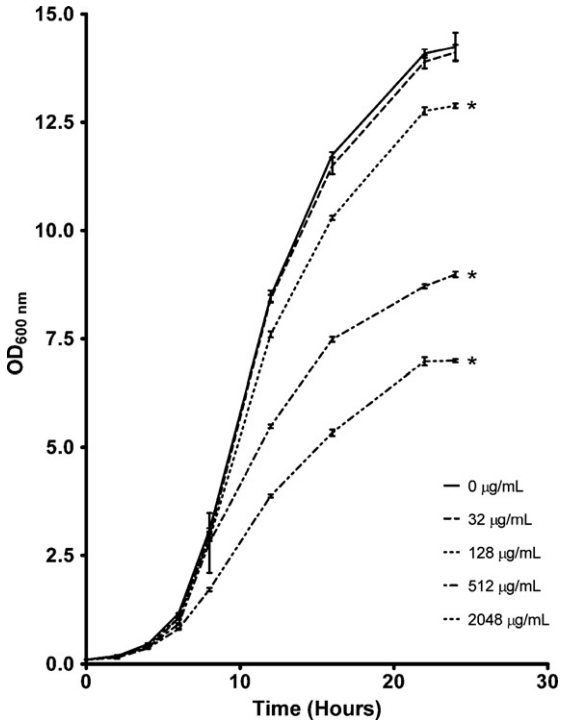

Fig. 1. In vitro effect of tigecycline (TIG) on the growth of planktonic *Candida albicans*. Increasing concentrations of TIG were added to complete synthetic media (CSM) containing a standardised inoculum of *C. albicans*. Growth curves began to diverge after 8 h of incubation, most prominently with cells incubated in CSM containing 2048 μg/mL TIG. After 24 h, changes in the growth rates were statistically different ($P < 0.05$) at 128 μg/mL TIG (9.6% reduction) compared with cells incubated in drug-free CSM. The greatest reduction in growth rate was seen with 2048 μg/mL TIG (50.9% reduction compared with untreated control). * Indicates statistical significance compared with growth curve of untreated cells ($P < 0.015$).