Enhancement of the *in vitro* activity of amphotericin B against the biofilms of non-*albicans* *Candida* spp. by rifampicin and doxycycline

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The *in vitro* activity of amphotericin B (AMB) alone and in combination with rifampicin (RIF) and doxycycline (DOX) was tested against the biofilms of 30 clinical isolates of non-*albicans* *Candida* (NAC) species namely, *Candida parapsilosis*, *Candida krusei* and *Candida glabrata*. The killing activity of AMB at 10×MIC was significantly increased in combination with either antibiotic. With RIF, the killing activity increased by 20.6, 23.5 and 14% against the biofilms of *C. parapsilosis*, *C. krusei* and *C. glabrata*, respectively; with DOX, the killing activity increased by 30.64, 35.28 and 31.13%, respectively. Pre-exposure of the isolates to the same combinations significantly reduced the number of colonized cells in the biofilms by 20, 25.14 and 13.07% with RIF for *C. parapsilosis*, *C. krusei* and *C. glabrata*, respectively, and by 18.94, 24.52 and 29.15% with DOX, respectively. The data showed that combination of RIF or DOX with AMB enhanced the killing activity of the antifungal agent against biofilms of NAC species. Whether such an effect operates against biofilm-associated infections needs to be clarified by further *in vivo* studies.

**INTRODUCTION**

Non-*albicans* *Candida* (NAC) species have emerged as both colonizers and pathogens causing nosocomial fungal bloodstream infections. They cause 35–65% of all candidemias in the general patient population (Krcmery & Barnes, 2002). The most common NAC species are *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* and *Candida glabrata* (Pfaller et al., 2000; Krcmery & Barnes, 2002). The majority of *Candida* nosocomial septicaemia is derived from intravascular catheters, urinary catheters, prosthetic heart valves, cardiac pacemakers, silicon voice prostheses and endotracheal tubes (Douglas, 2003).

The resistance of NAC species to antifungals represents a major challenge for future empirical therapeutic and prophylactic strategies. Some NAC species are resistant to two or three antifungal agents, and this may severely limit therapeutic options. The situation becomes more complicated when the fungus exists in biofilms. Micro-organisms within biofilms are characterized by being highly resistant to antimicrobial agents and host defence mechanisms. Microbial cells that grow in biofilm are 10 to 1000 times more resistant to antimicrobial agents compared to planktonic cells (Costerton et al., 1999; Mah & O’Toole, 2001). The cells that detach from the biofilm have also been found to be more resistant to antimicrobial agents than the same cells grown in suspension (El-Azizi et al., 2005). Clinically important antifungal agents, including amphotericin B (AMB), were found to be less active against *Candida* in biofilms than against planktonic cells (Kumamoto, 2002; Lewis et al., 2002).

The addition of antibiotics such as rifampicin (RIF) and tetracycline to AMB has been shown to enhance the *in vitro* activity of the antifungal agent against yeast and filamentous fungi (Huppert et al., 1974; Edwards et al., 1980; Hughes et al., 1984). The efficacy of these combinations was also shown in an experimental animal model (Arroyo et al., 1977) and in humans (Christenson et al., 1987) as well. However, no data are available for such combinations against biofilms. In this study, we evaluated the *in vitro* combinations of AMB with RIF and doxycycline (DOX) against the biofilms of *C. glabrata*, *C. parapsilosis* and *C. krusei*.

**METHODS**

**Chemicals and antibiotics.** Unless otherwise indicated, all chemicals (analytical grade) were purchased from Sigma-Aldrich. The antibiotics AMB, RIF and DOX were also purchased from Sigma-Aldrich.
Micro-organisms. A total of 30 clinical isolates, 10 each of C. parapsilosis, C. krusei and C. glabrata isolated from bloods of patients with central venous catheters, were provided by the microbiology laboratories at St John’s Hospital and the Memorial Hospital, Springfield, Illinois. In these hospitals, C. parapsilosis, C. glabrata and C. krusei are the most common NAC species associated with blood stream infections, especially in patients with inserted vascular catheters. The isolates were identified to species level by using API 20 C AUX for yeast (bioMérieux). They were also screened for biofilm formation as previously described (El-Azizi et al., 2004).

In vitro susceptibility of the NAC species to AMB. The susceptibility of the tested isolates to AMB was determined by the broth microdilution method described in the Clinical and Laboratory Standards Institute M27-A2 guidelines (Clinical and Laboratory Standards Institute, 2002). The combinations of AMB with RIF and DOX were assessed by the broth microdilution checkerboard assay. The means of the MICs for the antifungal agent alone and in combination with the antibiotics were calculated for each Candida species.

Susceptibility of the biofilms of Candida spp. to AMB, RIF and DOX alone and in combination. To form biofilms, yeast nitrogen base (YNB) medium (Difco) supplemented with 50 mM glucose was used. Inoculum suspensions (200 μl) of YNB medium containing 1 × 10^6 c.f.u. micro-organisms ml^-1 were delivered to flat bottom 96-well polystyrene plates (Falcon no. 353072; Becton Dickinson). After 48 h incubation at 37 °C, the supernatants containing the planktonic cells were aspirated and the remaining biofilms were washed three times using PBS, pH 7.3. Fresh YNB medium (200 μl) containing AMB (at 10 × MIC), RIF (32 μg ml^-1), DOX (8 μg ml^-1) or the antifungal agent combined with either one of the antibiotics was added to the plates. After 24 h incubation, plates were causiously aspirated and the intact biofilms were then quantified by using the tetrazolium sodium benzene sulfonic acid hydrate (XTT) reduction assay as described by Kuhn et al. (2002). Briefly, 200 μl lactate Ringers solution containing XTT (0.5 g l^-1) and menadione (1 μM) was added to the intact biofilms. The contents of the plates were mixed using a plate shaker (Lab-Line Instruments) for 5 min followed by incubation for 1 h at 37 °C in the dark. The intensity of the colour of the soluble formazan was then measured with a microplate reader (Multiscan Plus; Thermosan Systems) at 490 nm and compared to that of drug-free wells. For confirmation, haemocytometer and trypan blue exclusion were used parallel to the XTT colorimetric assay.

Standard curves, which correlate the organism number to the colorimetric signal, were constructed for each of the tested isolates. The pattern of the curves was not the same with the different strains of the same species because different strains metabolize the substrate with different capability. A linear relationship was observed between 10^5 and 10^9 cell ml^-1 with most of the tested isolates.

Post-antibiotic effect of AMB alone and in combination with RIF or DOX on biofilms of Candida spp. Fresh cultures on Sabouraud agar medium were harvested in polypropylene centrifugation tubes containing 20 ml PBS. The tubes were supplemented with AMB, RIF, DOX or with the antifungal agent combined with either one of the antibiotics at the same concentrations used in the previous experiment. The contents of the tubes were vigorously mixed and incubated at 37 °C for 90 min in a water bath shaker. The suspensions were then centrifuged at 8000 r.p.m. for 20 min and the supernatants were decanted. The cell sediments were washed four times by adding PBS followed by centrifugation to remove the antibiotics. Finally, the sediments were suspended in YNB medium and standardized by haemocytometer and trypan blue exclusion to contain 1 × 10^6 viable c.f.u. ml^-1. The biofilms were then formed and quantified as mentioned before. The viability of the cells within the biofilms was compared to that of the cells with no prior exposure to the antibiotics.

Effect of combination of AMB with DOX on the biofilms of C. krusei on a vascular catheter model. We used the in vitro polypropylene disc model as previously described (El-Azizi et al., 2004) to visualize the effect of combinations of AMB with DOX on a biofilm of C. krusei by using scanning electron microscope (SEM). The model consists of 3.25 cm^2 polypropylene disc designed to include two samples inserts. The first insert, 2 cm long, can hold a 0.5 cm^2 vascular catheter segment and the second insert, 0.75 cm^2 long, located in the centre, can hold a urinary tract catheter segment. After placing the vascular catheter segments in the sample inserts, the discs were sterilized with ethylene oxide and placed in sterile 4 × 4 multi-well plates. Culture suspension of clinical isolates of C. krusei (CK07) in YNB pre-exposed to AMB (10 × MIC) alone or in combination with DOX (8 μg ml^-1) was standardized to contain 1 × 10^6 c.f.u. ml^-1 as mentioned before. One millilitre of the suspension was then added to each well. After 48 h incubation at 37 °C without shaking, the segments were washed three times with PBS and prepared for SEM as described by Marrie & Costerton (1984). Briefly, they were fixed in glutaraldehyde in 0.1 M cacodylate buffer containing 0.15 ruthenium red for 3 h at 4 °C. They were then rinsed in fresh 0.1 M cacodylate buffer for 10 min (repeated three times) and post-fixed in 1.5 % osmium tetroxide for 1 h. They were dehydrated in a series of aqueous ethanol solutions (30–100 %) and dried by a critical point dryer (Autosamdr1) with CO2. The specimens were mounted on aluminium stubs with silver paste, allowed to dry for 3 h and then coated with gold/palladium using a cool-sputter coater E5100 II (Polaron Instruments). The segments were then examined in a SEM (S-500; Hitachi) at 20 kV.

Statistical analysis. Each experiment was performed in quadruplicate and the mean and the s.d were calculated. One-way analysis of variance (ANOVA) was used to determine the differences between various treatments. Tukey’s pair comparison test was used at the chosen level of probability (P<0.05) to determine significance difference between means.

RESULTS AND DISCUSSION

In vitro susceptibility of the NAC species to AMB

AMB showed good activity against all isolates with the minimum fungicidal concentration equal to or onefold higher than the MICs (Table 1). RIF and DOX, on the other hand, did not show any activity against the isolates up to the maximum concentration tested (512 μg ml^-1). When combined with AMB, both antibiotics enhanced its in vitro antifungal activity (Table 2). RIF enhanced the antimicrobial activity of AMB with all isolates except one isolate of C. glabrata, while DOX showed the same effect with the exception of one isolate of C. krusei. RIF reduced the MICs of AMB by onefold to twofold while the reduction was onefold to threefold in combination with DOX. Many studies showed these antibiotics to enhance the in vitro antifungal activity of AMB against Candida and fungi (Lew et al., 1977; Lou et al., 1977; Hughes et al., 1984). AMB binds to sterols in the fungal cell membrane and increases permeability (Kinsky, 1970), allowing entry and subsequent interference with RNA synthesis by RIF (Medoff et al., 1972), and with protein synthesis by DOX (Battner & Vasquez, 1971).
Susceptibility of biofilms of Candida spp. to AMB, RIF and DOX alone and in combination

Based on their capability to adhere and form biofilm, we used wild-type strains of different Candida spp. isolated from bloods of patients with central venous catheters. Despite the fact that AMB showed good activity against all free cells in suspension, the situation was different with the biofilms. AMB alone at 10⁶ MIC was capable of killing only 5 to 9% of the cells in the biofilms (Fig. 1). However, in combination with RIF, the killing activity of AMB increased by 20.60 (P<0.0001), 23.50 (P<0.0001) and 14% (P=0.003) against the biofilms of C. parapsilosis, C. krusei and C. glabrata, respectively; with DOX, the killing activity increased by 30.64 (P<0.0001), 35.28 (P<0.0001) and 31.13% (P<0.0001), respectively. Candida biofilms were found to be 20- to 30-fold more resistant to AMB compared to planktonic cells (Baillie & Douglas, 1998; Ramage et al., 2001; Chandra et al., 2001). A number

Table 1. In vitro activity of AMB against the tested isolates

<table>
<thead>
<tr>
<th>Micro-organism (n)*</th>
<th>Value (µg ml⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>MIC range</td>
<td>MIC₅₀</td>
<td>MIC₉₀</td>
<td>MFC range</td>
<td>MFC₅₀</td>
<td>MFC₉₀</td>
</tr>
<tr>
<td>C. parapsilosis (10)</td>
<td>0.50–1.0</td>
<td>0.50</td>
<td>1.0</td>
<td>0.50–1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>C. krusei (10)</td>
<td>0.50–2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.50–2.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>C. glabrata (10)</td>
<td>0.50–1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.50–1.0</td>
<td>1.0</td>
<td>1.0</td>
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MFC, minimum fungicidal concentration.

* n refers to the number of tested isolates.

Table 2. In vitro combination of AMB with RIF and DOX against the tested isolates

<table>
<thead>
<tr>
<th>Micro-organism (n)*</th>
<th>Mean MIC (µg ml⁻¹) ± SD</th>
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</thead>
<tbody>
<tr>
<td>AMB</td>
<td>AMB + RIF</td>
<td>P value</td>
<td>AMB + DOX</td>
<td>P value*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis (10)</td>
<td>0.645 ± 0.406</td>
<td>0.187 ± 0.096</td>
<td>0.0220</td>
<td>0.150 ± 0.094</td>
<td>0.040</td>
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<tr>
<td>C. krusei (10)</td>
<td>0.708 ± 0.330</td>
<td>0.291 ± 0.102</td>
<td>0.0199</td>
<td>0.250 ± 0.136</td>
<td>0.017</td>
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<tr>
<td>C. glabrata (10)</td>
<td>0.475 ± 0.218</td>
<td>0.256 ± 0.146</td>
<td>0.0169</td>
<td>0.181 ± 0.075</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

* n refers to the number of tested isolates.

The difference between the mean MIC of AMB alone and in combination with the antibiotics is significant at P≤0.05.

Susceptibility of biofilms of Candida spp. to AMB, RIF and DOX alone and in combination

Based on their capability to adhere and form biofilm, we used wild-type strains of different Candida spp. isolated from bloods of patients with central venous catheters. Despite the fact that AMB showed good activity against all free cells in suspension, the situation was different with the biofilms. AMB alone at 10×MIC was capable of killing only 5 to 9% of the cells in the biofilms (Fig. 1). However, in combination with RIF, the killing activity of AMB increased by 20.60 (P<0.0001), 23.50 (P<0.0001) and 14% (P=0.003) against the biofilms of C. parapsilosis, C. krusei and C. glabrata, respectively; with DOX, the killing activity increased by 30.64 (P<0.0001), 35.28 (P<0.0001) and 31.13% (P<0.0001), respectively. Candida biofilms were found to be 20- to 30-fold more resistant to AMB compared to planktonic cells (Baillie & Douglas, 1998; Ramage et al., 2001; Chandra et al., 2001). A number

Fig. 1. In vitro activity of AMB in combination with RIF or DOX against biofilms of Candida spp. The concentration of AMB was equivalent to 10×MIC against each isolate, while the concentrations of RIF and DOX were 32 and 8 µg ml⁻¹, respectively. Black bars, AMB; grey bars, AMB+RIF; white bars, AMB+DOX. The asterisks indicate that the difference is significant at P<0.05 compared to AMB-treated samples.

Fig. 2. Post-antibiotic effects of AMB in combination with RIF and DOX on biofilm formation by Candida spp. The concentration of AMB was equivalent to 10×MIC against each isolate, while the concentrations of RIF and DOX were 32 and 8 µg ml⁻¹, respectively. Black bars, AMB; grey bars, AMB+RIF; white bars, AMB+DOX. The asterisks indicate that the difference is significant at P<0.05 compared to AMB-treated samples.
of mechanisms have been proposed to explain this resistance, including phenotypic changes resulting from a decreased growth rate, contact-dependent gene expression, interaction with the extracellular polymeric matrix (Douglas, 2003; Kumamoto, 2002) and poor penetration through the biofilm mass (Samaranayake et al., 1998; Dorocka-Bobkowska et al., 2003) and hence reduce the number of colonized cells within the biofilm.

**Effect of combination of AMB with DOX on biofilms of Candida spp. on a vascular catheter model**

The biofilm of a C. krusei (CK07) isolate, pre-exposed to AMB and DOX, on vascular catheter segment was visualized by SEM (Fig. 3). This showed a significant reduction of the number of adherent cells of C. krusei (CK07) in the biofilm treated with AMB and DOX compared to drug-free catheter and catheter treated with amphotericin alone. DOX was selected because it exerted better activity than RIF in combination with AMB against the tested Candida spp. in suspensions and biofilms.

Our data showed that combination of RIF or DOX with AMB enhanced the killing activity of the antifungal agent against the biofilms of NAC species in vitro. Whether such an effect operates against biofilm-associated infections needs to be clarified by further in vivo studies.

**REFERENCES**


