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RESEARCH ARTICLE

The Role of Amphotericin B Alone and in Combination with Different Antibiotics and Antifungals on Biofilms Produced by *Candida* Species

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The Role of Amphotericin B Alone and in Combination with Different Antibiotics and Antifungals on Biofilms Produced by Candida Species

SUMMARY

Biofilm formation by Candida species is highly resistant to commonly used antifungal agents and is difficult to treat. Therefore, this study focused on effectiveness of combination therapy against Candida biofilms. The antimicrobial activities of amphotericin B (1 µg/ml or 10 µg/ml) alone or in combination with various antibiotics (doxycycline (20 µg/ml), tigecycline (20 µg/ml), colistin (30 µg/ml), rifampicin (120 µg/ml), ciprofloxacin (20 µg/ml)) or antifungals (clotrimazole (2.5 µg/ml), anidulafungin (10 µg/ml), caspofungin (10 µg/ml), itraconazole (2.5 µg/ml) and fluconazole (10 µg/ml)) were investigated against fungal biofilms produced by C. albicans SC5314, C. tropicalis ATCC 750 and C. parapsilosis ATCC 22019. Fungal viability was monitored by culture (colony-forming unit (CFU). According to the results, rifampicin and ciprofloxacin enhanced the activity of amphotericin B (10 µg/ml). Among the antifungals, clotrimazole displayed the most significant effect in combination with amphotericin B (10 µg/ml), especially against C. parapsilosis biofilms.

Consequently, combinations of amphotericin B and antibiotic or antifungal could be a promising option for the treatment of Candida biofilms.

Key Words: *Candida, Biofilm, Amphotericin B, Clotrimazole, Antifungal combinations, Antibiotics combinations.*

Amfoterisin B'nin Tek Başına ve Çeşitli Antibiyotik ve Antifungallerle Birlikte Candida Biyofilmleri Üzerine Etkisi

ÖZ

Biyofilm oluşumu Candida türlerinde antifungallere daha dirençli olmalarını sağladığından tedavisi güç enfeksiyonlara neden olmaktadır. Bu amaçla, bu çalışmada Candida biyofilmlerine karşı kombinasyon tedavisinin etkinliği araştırılmıştır. Amfoterisin B'nin (1 µg / ml veya 10 µg / ml) tek başına veya çeşitli antibiyotiklerle [(doksisiklin (20 µg / ml), tigesiklin (20 µg / ml), kolistin (30 µg / ml), rifampisin (120 µg / ml), siprofloksasin (20 µg / ml)] veya çeşitli antifungaller [(klotrimazol (2.5 µg / ml), anidulafungin (10 µg / ml), kaspofungin (10 µg / ml), itrakonazol (2.5 µg / ml), flukonazol (10 µg/ml)] ile kombinasyonlarının C. albicans SC5314, C. tropicalis ATCC 750 ve C. parapsilosis ATCC 22019 tarafından üretilen biyofilmlere karşı etkinlikleri araştırılmıştır. Sonuçlarımız rifampisin ve siprofloksasinin, amfoterisin B'nin (10 µg / ml) aktivitesini arttırdığını, antifungaller arasında ise klotrimazol ve amfoterisin B (10 µg / ml) kombinasyonunun özellikle C. parapsilosis biyofilmlerine karşı etkili olduğunu göstermiştir. Sonuç olarak, amfoterisin B ve antibiyotik veya antifungal kombinasyonları Candida spp. biyofilmlerinin tedavisi için ümit verici bir seçenek olabileceği düşünülmüştür.

Anahtar Kelimeler: *Candida, Biyofilm, Amfoterisin B, Klotrimazol, Antifungal kombinasyonu, Antibiyotik kombinasyonu.*

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INTRODUCTION

Candida species are opportunistic pathogens that reside in the human oral cavity, vagina, and gastrointestinal tract natural microbiota (Cho et al., 2014; Kumamoto, 2011). Infections caused by *Candida* species range from superficial infections to invasive infections including candidiasis and endocarditis, that frequently occur in immunocompromised and hospitalized patients. Drug abuse, organ transplantation, surgery, burns, and malignancies are major risk factors for invasive *Candida* infections and thus affect morbidity and mortality rates. *Candida albicans* is identified as the predominant pathogen in *Candida* species infections. Nevertheless, recently non-*Candida albicans Candida* (NCAC) species, such as *C. tropicalis* and *C. parapsilosis,* are increasing in prevalence (Diba et al., 2018; Fesharaki et al., 2013; Yesilkaya et al., 2017).

Candida species biofilms are virulence factors that promote infection especially when the host defense system is impaired during treatment. Microorganisms form biofilms as a survival strategy. Biofilm embedded microorganisms possess resistance to both antimicrobial agents and host immune responses when compared to their planktonic forms. Antimicrobial resistance is mainly due to low penetration of antibiotics into biofilm matrix, low oxygen and nutrient concentrations, and expression of biofilm specific genes (Taff et al., 2013). *Candida* species can cause life-threatening problems by forming biofilms on the surfaces of medical devices such as implants, heart valves, catheters, and ocular lenses (Kojic and Darouiche, 2004). Moreover, *Candida* biofilms pose a significant risk in cystic fibrosis (Chotirmall et al., 2010; Williams et al., 2016). Previous studies have shown that 60-70% of the *Candida* isolates from different clinical materials produce biofilm (Tellapragada et al., 2014).

The absence of appropriate antifungal therapy is a major contributor to the increasing mortality, as well as hospital length of stay and cost of the treatment, in *Candida* infections. One of the most preferred antifungal agents in the clinical practice is amphotericin B. Amphotericin B is a polyene class antifungal that acts by binding to ergosterol in the cell membrane. Susceptibility studies indicate that *Candida* biofilms may be up to 1000-times more resistant than planktonic cells to antimicrobial agents (Tobudic et al., 2012), resulting in potentially high toxicity to host cells (Mazu et al., 2016). Therefore, new drug strategies, therapies and synergistic drug combinations are required to combat biofilm-related *Candida* infections (Taff et al., 2013). The aim of this study was to evaluate the *in vitro* effects of antibacterial agents and traditional antifungals, both alone and in combination with amphotericin B against mature biofilms produced by *C. albicans* SC5314, *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 22019.

MATERIALS AND METHODS

Strains and growth conditions

The three most common biofilm-forming *Candida* species, reference isolates *C. albicans* SC5314 (ATCC MYA-2876), *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 22019 were used in this study. All three isolates are susceptible to the antifungals used in this study. Isolates were sub-cultured from frozen stocks onto Sabouraud dextrose agar (SDA, Difco, Sparks, MD, USA) plates and incubated at 30°C overnight to generate cultures for use in the following experiments. Yeast extract peptone dextrose (YPD, Sigma-Aldrich, St. Louis, MO, USA) agar and broth, and Roswell Park Memorial Institute (RPMI, Sigma-Aldrich, St. Louis, MO, USA) medium, supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich, St. Louis, MO, USA), was used in the biofilm assays.

Antimicrobial agents

Amphotericin B deoxycholate (purity; 99,8%, Bristol-Myers Squibb, New York, USA), clotrimazole (purity; 99,97 %, Bristol-Myers Squibb, New York, USA), fluconazole (purity; 99,8%, Pfizer, New York, USA), anidulafungin (purity; 98,8%, Pfizer, New York, USA), caspofungin (purity; 100%, Merck Sharp Dohme, Kenilworth, NJ, USA), itraconazole (purity; 100%, Sigma Aldrich, St. Louis, MO, USA), doxycycline (purity; 98,9 %, Kocak Pharma Ilac, Turkey), tigecycline (purity; 99,7%, Wyeth Pharmaceuticals, Madison, NJ, USA), colistin (purity; 100%, Sigma Aldrich, St. Louis, MO, USA), rifampicin (purity; 99,99%, Kocak Pharma Ilac, Turkey) and ciprofloxacin (purity; 99,99%, Kocak Pharma Ilac, Turkey) were obtain from the manufacturers. Stock solutions were prepared at 1280 mg/L for the antifungals and 5120 mg/L for the antibiotics, according to Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2006; CLSI 2012; CLSI 2014) and stored at -80°C for up to 6 months. The final concentrations of antimicrobial agents used for biofilm assay were their peak serum concentration (Cmax) values after intravenous drug administration. Antifungal agents' susceptibility was performed by broth dilution according to CLSI recommendations. All strains were determined as sus-

ceptible to anidulafungin (≤2 µg/ml), caspofungin (≤2 µg/ml), itraconazole (<0.125 µg/ml) and fluconazole (<8 µg/ml). *C. parapsilosis* ATCC 22019 was used as the susceptibility test control strain (CLSI, 2012).

Biofilm formation

Biofilms were formed in microtiter plates wells as previously described by Ramage et al. (2001) [18]. Briefly, YPD broth cultures were inoculated directly from overnight YPD agar cultures, and cultured for a further 24 h, in an orbital shaker at 30˚C. YPD broth cultures were centrifuged (about 3,000 rpm, 5-10 min) and the pellet washed twice with sterile physiological buffered saline (PBS), followed by resuspending in RPMI 1640 to a cellular density equivalent to $1x10^6$ cells ml^{-1}. Biofilms were formed by adding 200 µl of the standardized cell suspension into selected polystyrene flat-bottomed 96-well tissue culture microtiter plates wells (Greiner Bio-One, Kremsmuenster, Austria) and incubated for 48 h at 37˚C. After incubation, the supernatant was gently aspirated, and the non-adherent cells removed by washing the biofilms three times with PBS (Ramage et al., 2001).

Biofilm CFU assay

After obtaining the biofilms, wells were treated with antimicrobials at their Cmax values (doxycycline (20 µg/ml), tigecycline (20 µg/ml), colistin (30 µg/ml), rifampicin (120 µg/ml), ciprofloxacin (20 µg/ml), clotrimazole (2,5 µg/ml), anidulafungin (10 µg/ml), caspofungin (10 µg/ml), itraconazole (2,5 µg/ml) and fluconazole (10 µg/ml)). In addition, amphotericin B was tested at 1 µg/ml or 10 µg/ml due to interpatient variabilities of amphotericin B Cmax and areas under concentration-time curves (AUC) that were 8- to 10-fold greater for patients treated with liposomal amphotericin B than for patients treated with amphotericin B deoxycholate (Heinemann et al., 1997). Twofold concentration of tested antimicrobials Cmax values were prepared in RPMI. In wells containing combinations of amphotericin B and antimicrobials, 100 µl of each antimicrobial was placed directly onto the biofilm. In wells containing antimicrobials alone, 100 µl of RPMI was added together with the antimicrobial to provide equivalent volumes added to the biofilms. Control wells contained no antimicrobial agents. The biofilm cultures were incubated for an additional 24 h (Ramage et al., 2001). After 24 h of antimicrobial exposure, the biofilms were rinsed with 200 µl PBS, and the biofilms detached by vortexing (900 rpm) and sonication (both 5 min) (Meddison). The well contents were collected into sterile tubes and vortexed and sonicated again after the addition of 200 µl PBS. To enumerate the pathogen load cultures were serially diluted in sterile PBS and plated onto SDA using the drop plate method. Plates were incubated at 37 °C for 24 h and colonies counted and expressed as CFU (Tavernier et al., 2017).

Statistical analysis

All experiments were performed in triplicate in two separate sets of experiments. All data are expressed as mean values with corresponding standard deviations. One-way ANOVA and Bonferroni's Multiple Comparison tests were used to compare the differences between biofilms and a p-value of <0.05 was considered statistically significant.

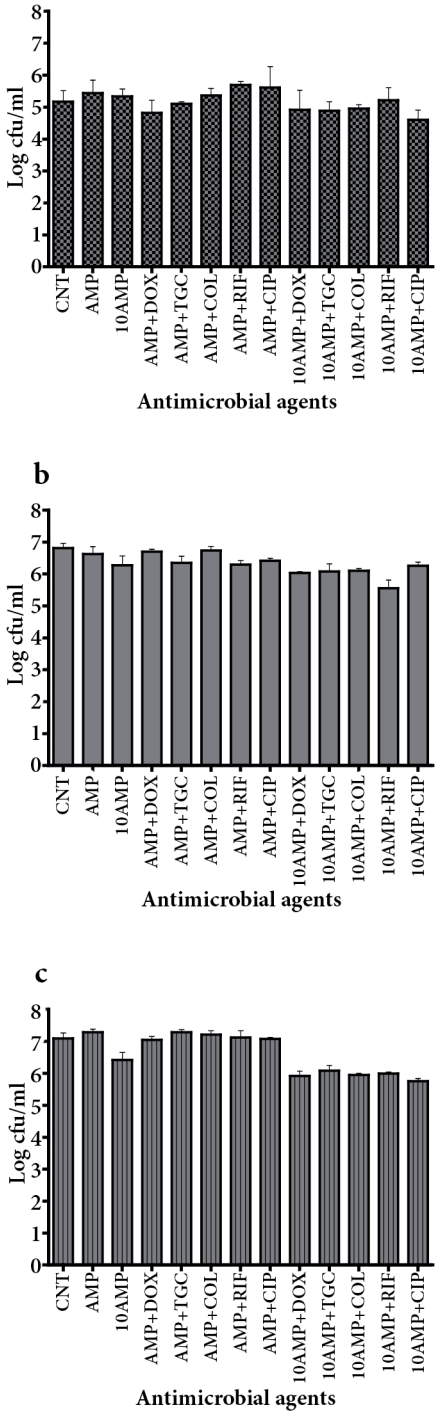
RESULTS

Amphotericin B and antibiotic combinations against *Candida* spp. biofilms

The antimicrobial effects of amphotericin B at two different concentrations (1 µg/ml or 10 µg/ml) alone and combination with antibiotics were evaluated. We observed that 10 µg/ml amphotericin B and ciprofloxacin combinations had better antimicrobial activity against *C. albicans* and *C. tropicalis* biofilms ($p<0.05$) than other combinations (Figure 1 a, c). In addition, the combination of amphotericin B (10 µg/ml) and rifampicin was the most effective in reducing *C. parapsilosis* biofilms viability (Figure 1 b). Furthermore, amphotericin B (10 µg/ml) with all antibiotic combinations displayed greater than one log reduction for each combination against *C. tropicalis* biofilms (Figure 1 c).

Amphotericin B and antifungal combinations against *Candida* spp. biofilms

The antibiofilm activities of amphotericin B alone and in combination with antifungals against *Candida* spp. was also evaluated at two different concentrations (1 µg/ml or 10 µg/ml). The most remarkable result was that clotrimazole was the most effective antifungal in combination with amphotericin B (10 µg/ml) against *C. tropicalis* and *C. parapsilosis* biofilms (Figure 2 b, c). Furthermore, clotrimazole and caspofungin alone were the most effective antifungals against *Candida* biofilms. However, there was no significant reduction in *C. albicans* biofilms with any of the antifungals or combinations studied (Figure 2 a).



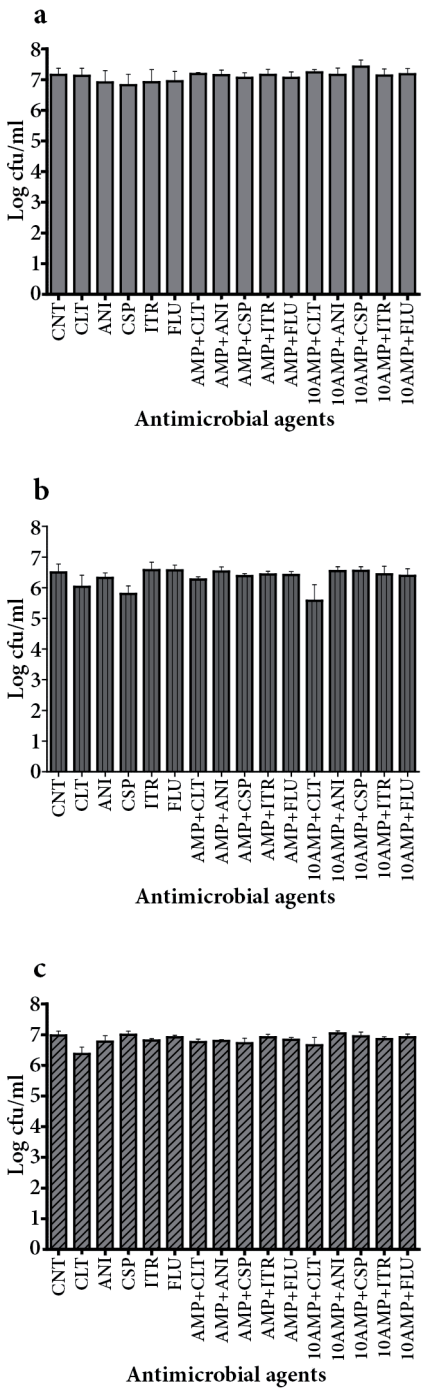
CNT: Control, AMP: Amphotericin B, DOX: Doxycycline, TGC: Tigecycline, COL: Colistin, RIF: Rifampicin, CIP: Ciprofloxacin

Figure 1. Amphotericin B and antibiotic combinations against *Candida* spp. biofilms. Average number of cfu of the microorganisms' recovered from biofilms were shown

a) *C. albicans* biofilms

b) *C. parapsilosis* biofilms

c) *C. tropicalis* biofilms



CNT: Control, AMP: Amphotericin B, CLT: Clotrimazole, ANI: Anidulafungin, CSP: Caspofungin, ITR: Itraconazole, FLU: Fluconazole

Figure 2. Amphotericin B and antifungal combinations against *Candida spp*. biofilms. Average number of cfu of the microorganisms' recovered from biofilms were shown

a) *C. albicans* biofilms

b) *C. parapsilosis* biofilms

c) *C. tropicalis* biofilms

DISCUSSION

In recent years, *C. albicans* and NCAC infections have been steadily increasing particularly in the patients with risk factors such as immunosuppression or drug abuse (Diba et al., 2018; Fakhim et al., 2017b, Fesharaki et al., 2013; Lamoth et al., 2018; Yesilkaya et al., 2017). Also, most likely due to the over prescription of antifungals, an important shift in infection rate with *C. albicans* to NCAC has occurred. The emergence of new multidrug-resistant NCAC strains such as *C. auris* become a significant threat worldwide (Fakhim et al., 2017a; Lamoth et al., 2018). Amphotericin B, fluconazole and echinocandins are most commonly prescribed in fungal infections (Lamoth et al., 2018). However, since *Candida* species are resistant to antifungals, studies to identify alternate treatment options such as combinational therapies, has gained significance (Fakhim et al., 2017a, Fakhim et al., 2017b).

According to a physiologically based pharmacokinetic model, plasma concentrations of drugs can vary with time due to different rates of absorption, distribution, metabolism and excretion (ADME) (Zhao et al., 2011). Pharmacokinetic drug interaction profiles are important to assess *in vivo*, as well as pharmacokinetic parameters such as Cmax and AUC when drug combinations are applied in clinical cases. Based on the clinical importance of these parameters, the average plasma concentrations were used to assist in understanding these interactions in this study.

The polyene antifungal agent amphotericin B, is the most reliable and broad-spectrum therapeutic agent for invasive fungal infections, including candidiasis and biofilm infections (Hamill, 2013; Touril et al., 2018). Since, yeasts are much more resistant to antifungal drugs in biofilms, high, frequently host cell toxic concentrations of amphotericin B are needed to destroy *Candida* biofilms or to decrease the cell number (Laniado-Laborin and Cabrales-Vargas, 2009). In order to avoid side effects and toxicities of amphotericin B, different strategies have been developed, including various drug formulations, combinational therapies (Aversa et al., 2017; Spader et al., 2019; Touril et al., 2018). Therefore, in this study we combined representative antifungal and antibacterial agents with amphotericin B to determine the effect of combinations on *Candida* biofilms.

According to the results, amphotericin B and antibiotic combinations demonstrated that rifampicin and ciprofloxacin can enhance the activity of amphotericin B against *Candida* biofilms. The activity of rifampicin against fungal RNA polymerase, as well as bacterial RNA polymerase, was previously identified (Del et al., 2011). Ciprofloxacin, that affects bacterial DNA gyrase, was previously demonstrated to interact pharmacodynamically with antifungal agents by altering their fungal growth-inhibitory activities, similar to rifampicin (Stergiopoulou et al., 2011). Furthermore, Stergiopoulou et al. (2008) has shown that low dose ciprofloxacin may increase pore formation induced by amphotericin B on fungi cell wall, thus lead synergistic effect. In this study the most effective combination against *C. parapsilosis* biofilm was amphotericin B and rifampicin. The enhanced antifungal activity observed with the combination of amphotericin B, ciprofloxacin and rifampicin, may be explained by more effective fungal cell penetration.

Previous studies have demonstrated that colistin alone affected the antifungal cell membrane at high concentrations (Schwartz et al., 1972). Colistin may have increased antifungal efficacy if given at lower concentration in combination with amphotericin B (TeixeirSantos et al., 2016). However, there is no study evaluating efficacy of this combination on *Candida* biofilms. Several studies evaluating doxycycline and tigecycline antifungal effectivity against *C. albicans* biofilms have indicated that addition of amphotericin B increased antifungal activity of these drugs (Hacioglu et al., 2018; Miceli et al., 2009). However, these studies were performed with high concentrations of antibiotics. The novelty of our study was to use plasma concentrations, not toxic levels of antibiotics. We observed that colistin, tigecycline and doxycycline had significant effects especially on *C. tropicalis* biofilms, even at lower plasma concentrations.

According to a study investigated amphotericin B (1 µg/ml) and caspafungin combination, there was no synergistic interactions against *C. albicans* biofilm (Tobudic et al., 2010). Similarly, amphotericin B-fluconazole and amphotericin B-caspafungin combinations against *C. albicans* biofilm have been determined as indifferent (Bachmann et al., 2003). In this study, amphotericin B and antifungal combinations except clotrimazol have not shown any significant result. An azole antifungal clotrimazole, which is widely used as a topical treatment for candidiasis, has become an area of intense research for the treatment of invasive fungal infections in select, high-risk patient populations (Crowley and Gallagher, 2014). Interestingly our results showed that clotrimazole displayed antifungal activity similar to caspofungin against NCAC, both alone and in combination with amphotericin B. To our knowledge this is the first report of clotrimazole having antibiofilm activity against NCAC biofilms.

Since microbial biofilms are highly resistant to antimicrobials, even a 1-log reduction in the number of microorganisms is very important in therapy. Consequently, it was determined that nucleic acid inhibiting antibacterial compounds enhance amphotericin B's antibiofilm activity at plasma concentrations. In addition, clotrimazole, an imidazole antifungal agent, destroyed biofilms more effectively than triazole antifungals, fluconazole and itraconazole. As expected, an echinocandine antifungal caspofungin alone was more effective on *Candida* biofilms than other antifungals. In conclusion, selected drug combinations could be potential alternatives for the current therapeutic management of fungal infections, but to date remain in the laboratory experimental phase. Further combinations need to be tested to confirm the potential synergistic effects between these antibiotics and antifungals at different concentrations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, finan-cial or otherwise.

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