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Anti-Biofilm Strategies: How to Eradicate *Candida* Biofilms?

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Abstract: In nature, microorganisms prefer to reside in structured microbial communities, termed biofilms, rather than as free-floating planktonic cells. Advantageous for the microorganisms, but disadvantageous for human health, is the increased resistance/tolerance of the biofilm cells to antimicrobial treatment. In clinically relevant biofilms, *Candida albicans* is one of the most frequently isolated microorganisms in biofilms. This review primarily elaborates on the activity of the currently used antimycotics against *Candida* biofilms, the potential of antifungal lock therapy and sheds more light on new promising compounds resulting from the gradual shift of anti-biofilm research activities to natural products, plants and their extracts.

Keywords: *Candida*, antifungal therapy, lock therapy, novel anti-biofilm agents.

INTRODUCTION

One of the reasons for the growing frequency of hospital acquired *Candida* bloodstream infections is the increasing use of immunosuppresive therapy in cancer and transplant patients, which leads to breakdown of the barrier between the gut and bloodstream [1]. *Candida* cells, like many other microbial organisms, are able to adhere to and colonize surfaces of medical devices, like central venous catheters, voice prostheses, intrauterine devices and prosthetic joints, among others, resulting in the development of a biofilm. Evidence for the occurrence of *Candida* biofilms on surfaces comes from various *in vivo* studies, in which the devices are examined upon removal out of the patients, or from animal model systems. Techniques such as scanning electron microscopy, confocal laser scanning microscopy and echocardiography (to demonstrate the occurrence of biofilms on heart valves) can be used for biofilm visualization [2-4].

Infections due to the presence of fungal biofilms are a major clinical concern as these structured microbial communities, embedded in an extracellular matrix, are characterized by increased resistance to antifungal therapy [5]. In many cases, the implant has to be removed in order to cure the infection. A combined action of different mechanisms is believed to contribute to increased resistance: (i) amplified expression of efflux pumps, (ii) a changing sterol composition in the membrane, (iii) limited diffusion of molecules through the extracellular matrix and (iv) the presence of persisters in the biofilm, which are able to tolerate high concentrations of antimycotics [6]. Interestingly, these persisters are not mutants but rather phenotypical variants of wild type cells [7]. Until now, the molecular basis of persistence in *C. albicans* biofims is not known [8].

This review focuses on the treatment options for biofilm associated *Candida* infections, as well as on novel anti-biofilm compounds or therapy strategies.

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CURRENT THERAPEUTIC OPTIONS

The current treatment options for fungal biofilm-related infections are very scarce due to the intrinsic increased tolerance of biofilms to antimycotics. In the mid 1990s, *C. albicans* biofilms were found to be resistant to the majority of the antifungal agents [9]. Patients with fungal biofilm-infected devices are rarely cured with mono-antifungal therapy and affected devices generally need to be removed [10-15]. Percutaneous vascular catheters may be removed quickly. However, the removal of infected heart valves, joint prostheses, central nervous system shunts and other implanted medical devices is problematic because these implants generally have a life-supportive function. Hence, succesful treatment, thereby retaining the implanted devices, are urgently needed in clinical practice.

Conventional Antimycotics

It has been more than a decade since the first reports were published regarding a near-total *in vitro* resistance of *Candida* biofilms to antifungal agents [9,16,17]. To this end, a variety of *in vitro* biofilm model systems have been developed including *in vitro* biofilm formation on the surface of small catheter material (polyvinyl chloride) discs, on denture acrylic strips or on silicone elastomer discs [17-19]. The reader is directed to the review by Coenye *et al.* in this special issue of Open Mycology for more information in this regard.

Azoles

An early report by Hawser and Douglas investigated the susceptibility of *Candida* biofilms grown statically on small polyvinyl chloride discs, to a number of clinically relevant antifungal agents. They demonstrated that fluconazole (a triazole) was most potent, while amphotericin B showed only moderate activity against biofilm cells, similar to the effect of itraconazole (a triazole), ketoconazole (an imidazole) and flucytosine. Although fluconazole proved to be the most effective agent against *Candida* biofilms, the concentration corresponding to 50% inhibition was 28 to 38

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times higher than the relevant MICs [9]. However, in 2004, *in vitro* mature *C. albicans* biofilms were found to be highly resistant to fluconazole and miconazole (an imidazole). In this study, biofilms were grown on denture acrylic discs in a constant depth film fermentor and maintained with artificial saliva to simulate biofilm formation in the oral cavity. Early phase *C. albicans* biofilms (2-6h) proved to be susceptible to fluconazole (256 mg/l) and miconazole (256 mg/l) in this model system, with a reduction of >83% and >99%, respectively, in viability after 24h biofilm exposure [20]. Very recently, the group of Coenye also demonstrated fungicidal activity of miconazole against mature *Candida* species biofilms [21]. In the latter study, *Candida* biofilms were grown statically on silicone discs (24h) whereafter they were exposed to 5 mM miconazole (corresponding to 2081 mg/l) during 24h. A substantial reduction, ranging from 89.3% to 99.1%, in the number of colony forming units recovered from the discs, was observed. The concentration miconazole applied in these *in vitro* experiments was higher than the commonly used therapeutic concentration, but is achievable during, for instance, antifungal lock therapy. Treatment of mature *Candida* biofilms with 5 mM fluconazole only resulted in a fungistatic effect [21]. Hence, depending on the *in vitro* system used, data on biofilm susceptibility differ substantially.

The azole antifungals were discovered about 30 years ago and refinements to the azole class led to agents with a triazole at their core. As the first-generation triazoles fluconazole and itraconazole have limitations related to their spectrum of antifungal activity and their tolerability, there have been efforts to develop new triazoles that address these limitations and have led to the regulatory approvals of voriconazole (approved by the FDA in 2002) and posaconazole (approved by the FDA in 2006). However, these newer azoles are not active against *C. albicans* or *C. parapsilosis* biofilms formed on silicone elastomer discs [22].

Polyenes

In line with the above data, Kuhn and coworkers confirmed the resistance of mature *C. albicans*, and also of *C. parapsilosis* biofilms, to conventional agents such as fluconazole and amphotericin B. They however showed that the newer lipid formulations of amphotericin B (liposomal amphotericin B and amphotericin B lipid complex) exhibited inhibitory activities against mature *C. albicans* biofilms, grown on silicone elastomer discs. The antifungal concentration resulting in 50% reduction in metabolic activity of the biofilm [23] was equivalent to the respective MIC50-value for planktonic cells [19]. Recently it was shown that liposomal amphotericin B, at its MIC (0.5 μg/ml) is also able to eradicate *C. albicans* biofilms in a novel continuous catheter flow model [24]. Using a newly developed *in vivo* rabit biofilm model, Shuford and coworkers assessed the *in vivo* activity of amphotericin B and caspofungin (see echinocandin section below) against *Candida* biofilm growth on central venous catheters. They demonstrated that systemic therapy with either caspofungin or amphotericin B deoxycholate and combined with intraluminal lock therapy (see further), significantly reduced colony counts of *C. albicans* associated with catheters and even resulting in sterile catheters in many cases [25]. Another study demonstrated that amphotericin B lipid complex was able to sterilise catheters on which *C. albicans* biofilms were formed, using a rabbit model of catheter-associated candidal biofilm [26].

Like amphotericin B, the polyene nystatin is one of the antifungal drugs most commenly used to treat patients with oral fungal infections topically. Susceptibility testing of nystatin, revealed resistance in all *Candida* isolates examined by Khun and coworkers, when grown as biofilms [19]. However, incorporation of nystatin into a thin-film polymer on deture material reduced *Candida* biofilm formation with 70-80% whereas this was 50-60% in case of coating with amphotericin B. This novel thin-film coating with various antifungals effectively inhibits *C. albicans* biofilm formation and should be evaluated as a potential preventive therapy for denture stomatitis [27]. Very recently, De Prijck and coworkers investigated the effect of nystatin released from modified polydimethyl siloxane disk as a model for incorporating antifungals in medical devices against biofilm formation by *Candida* spp. Nystatin exhibited a concentration-dependent inhibitory effect on *Candida* biofilm formation in a microtiter plate but not in a Modified Robbins Device [28]. The small fraction of free released nystatin killed *C. albicans* biofilm cells in the limited volume of a microtiter plate well but not in the flow system [29].

Echinocandins

Kuhn and colleagues further demonstrated that caspofungin and micafungin, both members of the more recent class of echinocandin antifungals, are active against biofilm-associated *C. albicans* [19]. A few months after their report, Bachmann and colleagues published similar results regarding the activity of caspofungin against *C. albicans* biofilms. In this case, biofilms were formed in wells of microtiter plates [30]. More than 97% reduction of the metabolic activity of sessile cells was observed after treatment with caspofungin concentrations well within its therapeutic range (0.125 μg/ml) [31,32]. The antifungal activity of caspofungin on *Candida* biofilms was also studied in relation to the biofilm maturation age and the yeast susceptibility to fluconazole. It appeared that the activity of caspofungin applied at MIC dose against *Candida* biofilms formed on sections of silicone catheters was species-dependent (i.e. *C. albicans* or *C. parapsilosis*). Moreover, caspofungin activity at its MIC depends on maturation age of the *C. parapsilosis* biofilms. However, the inhibition of biofilm metabolic activity caused by 2 mg/l caspofungin (which is within the therapeutic range) was not species-specific, independent on the state of biofilm maturation and not affected by the yeast's resistance to fluconazole [33]. In line with the above activity of micafungin against *C. albicans* biofilm [19], another research group confirmed this activity against *C. albicans* biofilms, in this case grown on central venous catheter sections, and extended the observations to biofilms formed by *C. glabrata* and *C. parapsilosis* [34]. In contrast, Choi and coworkers showed that within the therapeutic concentration range of micafungin and caspofungin, both drugs are active against *C. albicans* and *C. glabrata* biofilms, but not against biofilms formed by bloodstream isolates of *C. tropicalis* or *C. parapsilosis* [35]. In conclusion, these inter-study variations highlight the differences in susceptibilities of *Candida* biofilms, which can be due to differences in the

biofilm-forming abilities of the *Candida* isolates tested or to the diversity of the biofilm models used.

In 2006, the FDA approved the new systemic echinocandin anidulafungin and two years later the first publication on its efficacy against *Candida* biofilms appeared [22]. Anidulafungin, like caspofungin and micafungin, showed enhanced potency against *C. albicans* biofilms formed on silicone elastomer discs at clinically relevant concentrations. The efficacy of caspofungin against *C. albicans* biofilms *in vivo* was further demonstrated in a model for central venous catheter-associated candidiasis in mice. Treatment with 0.25 μg/ml caspofungin (instilled in the catheter and allowed to dwell for 24h) significantly diminished the biofilm fungal load in the catheters and the dissemination to the kidneys compared with untreated controls [36]. Recently, the efficacy of anidulafungin against *in vivo* mature *C. albicans* biofilms was demonstrated using a newly developed rat model of catheter-associated candidiasis. The model was based on the avascular implantation of small polyurethane catheters challenged with *Candida* cells prior to their implantation in immunosuppressed rats. The model allowed the study of up to 10 biofilms at once, and the recovery of mature biofilms from 2 days after implantation. The adhering inoculum was adjusted to the standard threshold of positive diagnosis of fungal infection in materials recovered from patients. Wild type biofilms were mainly composed of hyphal cells, and they were unevenly distributed across the catheter length as observed in infected materials in clinical cases [10]. This model can be used to characterize the ability of antimicrobial agents to eliminate biofilms, and to evaluate the prophylactic effect of antifungal drugs and biomaterial coatings [37,38]. In the latter case, anidulafungin was daily administered intraperitoneally at 10 mg/kg for 7 days. More than 70% of explanted catheters from anidulafungin-treated animals contained fewer than $2\log_{10}$ colony forming units of *C. albicans* cells which was below the diagnostic threshold for catheter related infections [10]. In addition, 17% of the catheters from anidulafungin-treated animals were sterile [38]. In conclusion, *Candida* biofilms are intrinsically resistant to most antifungal drugs such as azoles (with the exception of miconazole). However, the lipid formulations of amphotericin B and the echinocandins do have activity against *Candida* biofilms.

Antifungal Combination Therapy

Efforts to successful treatment of *Candida* biofilm-associated infections are urgently needed in clinical practice. Combining antifungal drugs was recommended as a means to enhance efficacy in a variety of invasive infections including cryptococcosis, candidiasis, and aspergillosis. There are several foreseeable advantages to combination antifungal therapy, (i) a widened spectrum and potency of drug activity, (ii) more rapid antifungal effect, (iii) synergy, (iv) lowered dosing of toxic drug and (v) reduced risk of antifungal resistance. Utilizing agents with different mechanisms of action is a hallmark in current medical therapies in numerous medical disciplines, but of course one has to be cautious of some combinations as they may be antagonistic or clinically indifferent with additive side effects. Prior to the availability of echinocandins and azoles, the polyene antifungal agent amphotericin B was the major antifungal agent used in the management of invasive *Candida* infections. The high incidence of nephrotoxicity of amphotericin B led to the evaluation of combination therapies, to reduce the concentration and the toxicity required for treatment [39]. In 2002, Khun and coworkers demonstrated for the first time the unique activity of lipid formulations of amphotericin B and echinocandins against *Candida* biofilms [19]. Because *in vivo* and clinical studies are hard to perform, most of the reports below deal with *in vitro* studies of biofilm susceptibility following antifungal combination treatment. Synergism or antagonism of antifungal combinations is most commonly determined by the checkerboard method [40,41]. Bachmann and coworkers described in 2003 the indifferent interaction of either paired combination of the antifungals amphotericin B, caspofungin and fluconazole against biofilms of a single *C. albicans* isolate *in vitro*. They demonstrated further an antagonistic effect by time-kill experiments of high fluconazole doses and caspofungin [42]. The combination caspofungin and voriconazole did not provide either an enhanced activity compared with caspofungin alone against biofilms of 30 clinical invasive strains of *C. albicans* associated with human infection [43]. However, very recently, Tobudic and coworkers investigated the *in vitro* activity and synergism of the combination of amphotericin B and caspofungin on one hand, and amphotericin B and posaconazole on the other hand against *C. albicans* biofilm cells. They reported that the combination of amphotericin B/pozaconazole yielded synergism, whereas amphotericin B/caspofungin yielded indifferent interactions against biofilms of various *C. albicans* isolates [44]. The indifference of amphotericin B/caspofungin is in line with the previous data of Bachmann and coworkers [42]. In addition, Pai and coworkers performed studies of various antifungal combinations against simulated *Candida* endocardial vegetations [45,46]. Current medical management includes combination use of amphotericin B and flucytosine followed by prolonged suppression with fluconazole [47,48]. However, multidrug resistance to long-term suppressive therapy can develop [49]. The low incidence and high morbidity and mortality associated with this disease demands alternative research strategies to improve current care. Surgical removal of the infected valve is the most effective management strategy, although not always possible. In 2008 Pai and coworkers investigated the effects of combinations of flucytosine, micafungin and voriconazole against *Candida*-infected human platelet-fibrin clots in an *in vitro* endocarditis pharmacodynamic model. Single clinical bloodstream isolates of *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* were used. Voriconazole was identified to be the least active agent, while flucytosine had the greatest activity as single agent. Micafungin was superior to voriconazole for all species except *C. parapsilosis*. The combination of flucytosine/voriconazole was superior to either agent alone against *C. parapsilosis*. The combination of voriconazole/micafungin was inferior to the use of micafungin alone against *C. tropicalis*. The triple combination flucytosine/voriconazole/micafungin was not better than single or dual agents against any of the *Candida* spp. [45]. As previous studies demonstrated the poor activity of voriconazole and the superior activity of flucytosine and micafungin against *C. albicans* biofilms, Pai continued his research with the investigation of

the effects of flucytosine, liposomal amphotericin B and micafungin combinations against 2 *C. albicans* strains in the same endocarditis pharmacodynamic model. They studied the effects of the antifungals on 24h-old biofilms. By contrast with the previous study in 2008, they measured the antifungal effects only at one time point and used the standard definition of synergism. These data revealed that micafungin was uniquely active against mature *C. albicans* biofilms, while flucytosine was not [46]. This is in contrast to the data in the previous report, where flucytosine was markedly active against *C. albicans* biofilms [45]. The triple combination of flucytosine/liposomal amphotericin B/micafungin was superior to all other treatments for one isolate but not different from the dual combination liposomal amphotericin B/micafungin for the other isolate. Although no clear pattern of interaction between these agents was seen, the superior activity of micafungin against 24h-old *C. albicans* biofilms relative to flucytosine and liposomal amphotericin B was apparent for both isolates [46]. In conclusion, and supported by earlier reports [19], these data demonstrated the activity of echinocandins when used alone or in combination with polyenes against *C. albicans* biofilm.

In 2008, Uppuluri and coworkers demonstrated a synergistic effect of calcineurin inhibitors FK506 and cyclosporin A in combination with fluconazole against *C. albicans* biofilms in both *in vitro* experiments and an *in vivo* rat catheter model. These studies reveal that the drug synergism was mediated via direct inhibition of *C. albicans* calcineurin, which is known to contribute to fluconazole resistance in biofilms. [50].

Very recently, studies involving the combination of antibacterial agents with standard concentrations of antifungal agents against *C. albicans* biofilms were reported [51, 52]. In 2009, Miceli and coworkers determined the antifungal effect on *C. albicans* biofilms of doxycycline alone and in combination with standard combinations of amphotericin B, caspofungin and fluconazole. In this study, 128 μg/ml doxycycline combined with fluconazole and 512 μg/ml doxycycline combined with low doses of amphotericin B (≤ 0.25 μg/ml) demonstrated synergism. The paradoxical effect of high concentrations of caspofungin (16 μg/ml) was significantly attenuated in combination with 2048 and 512 μg/ml doxycycline. Other combinations and concentrations did not show significant positive effects against the biofilms [51]. Because of the interesting results of combination of antifungals with doxycycline, Ku and coworkers determined in 2010 the activity of other antibacterial agents, including azithromycin, tigecycline and vancomycin against *C. albicans* biofilms. Tigecycline was the most active agent against mature *C. albicans* biofilms and substantially inhibited the formation of the biofilms. Therefore they focused on the combination of tigecycline with amphotericin B, caspofungin and fluconazole. However, addition of tigecycline did not potentiate the effects of the antifungals tested and in some cases, inhibited their effects [52]. In another study, Shi and coworkers demonstrated the synergism between fluconazole and minocycline against fluconazole-resistant *C. albicans* isolates. The mechanism of action was suggested to be the enhancement of minocycline on fluconazole penetrating through the biofilm as well as interrupting the calcium balance instead of impacting on the uptake and efflux on fluconazole [53]. However, it should be mentioned that there are risks of inducing bacterial resistance by using the combination of high doses antibacterial agents with standard concentrations of antifungal agents against *C. albicans* biofilms.

Synergism and antagonism are *in vitro* concepts that are difficult to translate into clinical practice. Clinical studies are needed but hard to perform. Nevertheless, evidence of synergism of antifungal combination therapy *in vitro* might be the first step in establishing appropriate antifungal therapy.

Antifungal Lock Therapy

In case of implant-related infections, the guidelines for the treatment of candidiasis strongly recommend the removal or replacement of catheters and medical devices whenever feasible [14]. However, in many patients with biomaterial- or catheter-related *Candida* infection, removal and/or replacement of the infected device is difficult or a high risk. As an alternative strategy, antimicrobial lock therapy (ALT) has been proposed for the prevention and treatment of catheter-related bloodstream infections, often associated with biofilm formation [19]. The guidelines for the treatment of candidiasis also state that the role for antifungal lock solutions is not well defined. The ALT involves introducing 2 to 4 ml of a highly concentrated antimicrobial solution (100-1000x MIC or its usual target systemic concentration) into a catheter lumen and allowing the solution to dwell (lock) for a specific period of time while the catheter is not in use, in order to affect the biofilm and sterilize the lumen [54,55]. Microorganisms most frequently associated with catheter-related bloodstream infections are *Staphylococcus epidermis*, *S. aureus* and *Candida* species [56,57], and these microbes have been isolated together in biofilm-related polymicrobial infections [58]. The reader is directed to the review by Rizk *et al.* in this special issue of Open Mycology for more information on polymicrobial biofilms. A variety of antibiotics have been used as prophylactic antimicrobial catheter lock solution, including gentamycin, cefazolin/gentamycin, minocycline or cefotaxime, and were effective in preventing catheter related bacteraemia [59-63]. In the reviewed studies by Korbila and coworkers, there were no serious adverse effects, such as emergence of resistance or increased infectious complications found to be associated with the use of ALT [64]. However, Allon and coworkers are concerned about the use of antibiotics because of the potential development of resistance and the risk for systemic toxicity due to solution leak from the catheter lumen into the circulation [65]. Non-toxic anti-biofilm agents are therefore urgently needed.

In 2003, Raad and coworkers, investigated the *in vitro* activities of different flush solutions against *S. epidermis*, *S. aureus* and *C. albicans* biofilms grown on catheter surfaces. They used a Modified Robbins device [28] as *in vitro* model whereby the catheter segments were flushed for 18h with streptokinase, heparin, vancomycin, vancomycin/heparin, EDTA, minocycline or minocycline/EDTA [66]. EDTA is a chelator of calcium and magnesium with established anticoagulant activity and inhibitory activity against S*taphyloccoci* and *Candida* spp., which shows better results than heparin when used in flush solutions [66-70]. Minocycline is a broad spectrum tetracycline antibiotic. Only

EDTA resulted in partial reduction of catheter colonization. As previously reported, this study shows also the failure of vancomycin and heparin against catheter-related biofilms. The combination of EDTA with low minocycline concentration (0.1 mg/ml) resulted in a significant decrease in catheter colonization but combined with higher concentrations of minocycline resulted in complete eradication of *C. albicans* biofilms. These data support other reports involving the combination of minocycline and EDTA as effective lock solution against central-venous-catheter-associated biofilms of clinically relevant microorganisms in clinical trials [71,72] and a rabbit model [73]. Another study shows that tetrasodium EDTA applied for 21h to 25h reduced the biofilm formation of different strains, including *C. albicans* [74]. Based on *in vitro*, animal and clinical studies, for an ALT to be effective, a dwell time of at least 4h daily is often required [55,68,71,75,76]. This might not be feasible among patients who continuously require fluids and therapeutic agents through the catheters. As ethanol was shown to have only a dwell time for 1h [77], more studies involving the use of ethanol alone or in combination with other agents were reported. Maki and coworkers showed in addition that ethanol does not alter the mechanical properties of silicone and polyurethane catheters [77]. Although low concentrations of ethanol (1-6%) stimulate biofilm formation in some strains of *S. epidermis* [78], 10% ethanol significantly inhibited *Candida* biofilm formation *in vitro* with complete inhibition at concentrations ≥ 20%. In 2009, Balestrino and coworkers reported data involving a lock solution based on 60% ethanol. This *in vitro* study demonstrated the superior antimicrobial activity of 60% ethanol in contrast to 47.6% trisodium citrate in eradicating monomicrobial biofilms including *C. albicans* biofilms formed on silicone catheters. A 20 min 60% ethanol-treatment completely eradicated the 4- and 24h-old *C. albicans* biofilms [79]. These results are in line with an earlier report [80]. The advantage of using ethanol as a lock solution in addition to the antimicrobial activity, is the low cost and universal availability. There is no evidence of acquired resistance to concentrated ethanol despite the extensive and longstanding use as an antiseptic, nor are there any studies showing hypersensitivity related to ethanol administration. Preliminary reports suggest that ethanol as a lock solution can be used without severe side effects [81-84].

In 2007, Raad and coworkers performed a study involving different combinations of minocycline, EDTA and 25% ethanol. In this study, the triple combination of minocycline/ EDTA and a low concentration of ethanol (25%) was the only lock solution of the study that completely eradicated and completely prevented the regrowth of the biofilms of 2 *Candida* strains in various biofilm models. In addition this lock solution was more effective in rapidly eradicating the growth or regrowth as compared to other combinations of the agents [85].

Venkatesh and coworkers investigated the effects of different agents including ethanol on monomicrobial and polymicrobial biofilms of *C. albicans* and *S. epidermis* formed in a microtiter plate model. The minimal biofilm eradicating concentration causing 50% inhibition (MBEC50) for ethanol was 12.5%. This was the only agent that reduced the viability of *C. albicans* in polymicrobial biofilms [86]. These results are in line with previously reported data involving the effectiveness of higher concentrations of ethanol (25-70%) against biofilms [80,85].

The *in vitro* anti-biofilm activity of echinocandins has been previously demonstrated, however the potential of echinocandins used as a lock therapy is poorly documented. In 2008, Cateau and coworkers investigated the *in vitro* efficacies of caspofungin or micafungin catheter lock solutions on *C. albicans* biofilm growth. The results demonstrated that caspofungin or micafungin used as a lock solution significantly reduced at least by half the metabolic activity of intermediate (12h) and mature (5 days) biofilms of *C. albicans* and that the reduction was maintained after 48h [87]. So echinocandins may have a real anti-biofilm potential and could become important factors in the lock approach.

There are different reports involving liposomal amphotericin B as antifungal lock therapy against *C. albicans* biofilms in a catheter infection model. The first clinical relevant model of *C. albicans* biofilms-associated catheter infection was described by Schinabeck and coworkers in 2004. They evaluated the effectiveness of liposomal amphotericin B (3 mg per day) antifungal lock therapy compared to a fluconazole (3 mg per day) lock and untreated controls against *C. albicans* biofilms (3 days old) formed on silicone catheters in rabbits. The antifungal solutions were locked in the lumen of each catheter for 8h per day for 7 days after 3 days post infection. Results showed that catheters treated with liposomal amphotericin B were completely clear except for 1 or 2 small patches of dead *C. albicans* surrounded by minimal amounts of damaged matrix [88]. These results are in line with earlier reports with successful salvage of catheters infected with *C. albicans* by using amphotericin B deoxycholate antifungal lock therapy [89,90]. As in the case of these reports, the treatment period of amphotericin B may be extended to 10 to 14 days for complete eradication of fungal elements of the catheters. The fluconazole treatment resulted in less biofilm compared to the untreated controls, but the biofilm structure appeared similar to that of the controls, unlike the amphotericin B treatment. Increased concentrations of fluconazole may improve its efficacy as an antifungal lock solution. However, dissolving higher concentrations in this study was difficult. Even though fluconazole lock therapy is unable to sterilize the intraluminal surface of catheters, only 1 out of 7 catheter drawn blood cultures grew yeast after the treatment period. The fluconazole solution may only kill free-floating planktonic *C. albicans* cells released from the mature biofilm. Therefore fluconazole lock therapy may represent an effective suppressive therapy for preventing continuous seeding of the bloodstream, although the infection may return when the therapy is stopped [88].

A few years later, Shuford and coworkers evaluated caspofungin and amphotericin B deoxycholate against *C. albicans* biofilms in a rabbit catheter infection model [25]. Their study demonstrated the effectiveness of amphotericin B and more specifically caspofungin, in the combination of systemic and intraluminal lock therapy against *C. albicans* biofilms associated with intraluminal and extraluminal surfaces of catheters. However, the continuous lock with amphotericin B over the treatment duration of 7 days is not practical in a clinical setting, where catheters are placed to facilitate frequent delivery of medications and/or nutrition

and which is not possible if the catheter is continuously locked. In 2009, these data were confirmed in an independent study by Mukherjee and coworkers [26].

In conclusion, all these data demonstrate that liposomal amphotericin B lock therapy is an effective approach for treating *C. albicans* catheter-associated biofilms in a short treatment period. These findings may have significant clinical implications and warrant clinical evaluation.

Novel Anti-Biofilm Agents

The development of new anti-biofilm agents is urgently needed as the number of therapeutic options for *Candida* biofilm-related diseases is very small. There is a novel trend in the anti-biofilm research area towards the identification of natural products, plants and their extracts with anti-biofilm activity. In this respect, xanthorrhizol isolated from *Curcuma xanthorrhiza* [91] and the oil of *Boesenbergia pandurata* rhizomes [92] and *Ocimum americanum* [93] showed potent *in vitro* activity against *Candida* biofilms. In 2008, 30 plant oils were tested for their activity against *C. albicans* biofilms [94]. Peppermint, eucalyptus, ginger grass and clove oils resulted in a reduction in *C. albicans* biofilm formation. The main component of eucalyptus oil, 1,8-cineole, showed potent anti-biofilm activity against *C. albicans* biofilms [95]. The antifungal activity of tea tree oil has been recently studied by De Prijck and coworkers against *C. albicans* biofilms. The tea tree oil was released from modified polydimethyl siloxane disk as a model for incorporating antifungals in medical devices to prevent biofilm formation by *Candida* spp. The biofilm inhibition amounted to more than one log unit in the Modified Robbins Device [28] on disks impregnated with tea tree oil [96]. Also the strong antifungal activity of terpenes, which are major components of essential oils, has been described [97-99]. In 2008, 10 terpenic derivatives, corresponding to major components of essential oils, were tested for their activity against *C. albicans* biofilms. Almost all the studied terpenic derivatives showed anti-biofilm activity. However, carvacrol, geraniol and thymol exhibited the strongest activity. Moreover, these compounds also proved to be efficient against biofilms of *C. glabrata* and *C. parapsilosis* [100].

Chitosan recently proved to be active against *in vitro Candida* biofilms as well. Chitosan is a hydrophilic biopolymer that is industrially obtained by means of *N*-deacetylation of crustacean chitin. It was already known that chitosan exhibits antimicrobial activity against fungi, bacteria and viruses [101]. Now, it was determined that mature *C. albicans* and *C. parapsilosis* biofilms are susceptible to chitosan *in vitro*. Chitosan decreased the metabolic activity and survival of *Candida* species biofilms, with more than 95% killing of the sessile cells after 0.5h treatment with 2.5 mg/ml chitosan [102]. Also various saponins [103] and polyphenols [104] were shown to affect *C. albicans* biofilms. Saponins are secondary metabolites ubiquitously found in various plant species and specifically known for their hemolytic activity [103]. Polyphenols, extracted from green tea, also showed effects against *C. albicans* biofilms. Epigallocatechin-3-gallate, the most abundant polyphenol in a green tea extract, reduced the *C. albicans* biofilm metabolic activity with 80%. Further investigations indicated that impairment of the yeast proteasomal activity might be involved in the mode of action of tea polyphenols against *C. albicans* [104]. Bisbibenzyl compounds are exclusively found in liverworts and are known to exhibit antifungal activity [105-107]. In 2009 it was demonstrated that riccardin D, a macrocyclic bisbibenzyl isolated from the Chinese liverwort *Dumortiera hirsuta*, causes a remarkable reduction in the metabolic activity of *C. albicans* biofilm cells at concentrations that are not toxic for RPE1 or LO2 cell lines [108,109]. Furthermore, it was shown that riccardin D acts by down regulating the expression of *Candida* hyphae specific genes [109]. In addition, (R)-goniothalamin, the most abundant styryl lactone in the *Goniothalamus* genus (*Annonaceae* family), is active against *C. albicans* biofilms [110]. It was already reported that (*R*)-goniothalamin as well as its non-natural enantiomer (*S*)-goniothalamin, exhibit antifungal activity against human pathogenic fungi and have minimal toxic effects on mammalian cell lines [111].

Carbazoles are aromatic heterocyclic organic compounds first isolated from coal tar [112]. Carbazole alkaloids have been isolated form various plants and microorganisms. In a recently conducted compound screening, aimed at finding novel fungicidal compounds, a series of substituted carbazoles (N-alkylated 3,6-dihalogenocarbazoles) was identified. Besides their fungicidal activity against planktonic *C. albicans* and *C. glabrata* cells, some of them were also active against *Candida* biofilms grown in microtiter plates [113].

Besides echinocandins, various other naturally occurring peptides have been found with potent activity against *Candida* biofilms, including lactoferrin and histatins. Lactoferrin is an iron-binding glycoprotein, which is naturally present in human glandular secretions (milk, tears and salvia), and has a broad-spectrum antimicrobial activity against bacteria and fungi [114]. Venkatesh and coworkers observed that talactoferrin, human recombinant lactoferrin, significantly decreased the biomass and thickness of *S. epidermis* and *C. albicans* biofilms [86].

Histatins, a family of histidine-rich cationic peptides secreted by the major salivary glands in humans, especially histatin 5, possess significant antifungal properties [115]. A recent study, demonstrated that histatin 5 exhibits antifungal activity against biofilms of *C. albicans* and to a lesser extent against biofilms of *C. glabrata* developed on denture acrylic [116]. Naturally occurring antimicrobial peptides hold promise as therapeutic agents against pathogens such as *C. albicans* but numerous difficulties have slowed their development. They are difficult and expensive to produce in large quantities and are often sensitive to protease digestion [117]. The quest for new and improved antimicrobial peptides has led to the study of peptide mimetics. Synthetic analogs that mimic the properties of these peptides have many advantages and exhibit potent, selective antimicrobial activity [118]. In 2002, Stark and coworkers developed a new class of antimicrobial peptides that were originally designed to mimic transmembrane segments of integral membrane proteins and were tagged with lysine residues to facilitate solubilization in aqueous media. The peptides, designated kaxins, have a non-amphipathic hydrophobic core segment, which distinguishes them from many natural linear cationic antimicrobial peptides. They showed that placing all of the K residues on the N-terminus and generating all-D-

enantiomeric versions, in combination with decreasing the length of the hydrophobic segment, resulted in shorter (and therefore less expensive) peptides that generally displayed increased antimicrobial activity [119]. In 2006, Burrows and coworkers tested the antifungal activity of a subset of those peptides with a good antimicrobial activity without mammalian cell toxicity, and new deratives thereof. They performed different assays including the ability of 2 peptides (dF17-6K and dF21-10K) with the best antifungal activity to kill biofilms of *C. albicans* and *C. tropicalis* [120]. The amount of peptide required for eradication was less than the 30-2000x MIC required for small molecule antifungals to kill biofilms [121]. The amount of peptide may be reduced even further by attaching the peptides directly to the surface of biomaterials to prevent biofilm formation at the stage of initial attachment, as shown to be successful for long-chain hydrophobic polycations, which are mimics of antimicrobial peptides [122,123].

Based on the antifungal activity of β-peptides (β-amino acid oligomers) against planktonic *C. albicans*, Karlsson and coworkers demonstrated in 2009 that these β-peptides at a concentration near the minimum inhibitory concentration completely prevented planktonic cells to form a biofilm by a toxicity mechanism involving membrane disruption [124]. Recently, the fabrication of multilayered polyelectrolyte thin films that promote the surface-mediated release of an antifungal β-peptide was reported [125]. The films inhibited the growth of *C. albicans* on film-coated surfaces, resulting in a 20% reduction of cell viability after 2h and a 74% decrease in metabolic activity after 7h when compared to cells incubated on coated surfaces without β-peptide. In addition, β-peptide-containing films inhibited hyphal elongation by 55%. This approach could ultimately be used to coat the surfaces of catheters, surgical instruments, and other devices to inhibit drug-resistant *C. albicans* biofilm formation in clinical settings [125]. In addition, Hua and coworkers screened several series of antimicrobial peptide mimetics (with molecular weight <1000 Da) against oral *Candida* strains. One phenylalkyne and several arylamide compounds with reduced mammalian cytotoxicities were found to be active against *C. albicans*. These compounds demonstrated rapid fungicidal activity in liquid culture even in the presence of saliva, and demonstrated synergy with standard antifungal agents. When assayed against biofilms grown on denture acrylic, the compounds exhibited potent fungicidal activity. Repeated passages in sub-minimum inhibitory concentration levels did not lead to resistant *Candida*, in contrast to fluconazole. These results demonstrate the proof-of principle for the use of these compounds as anti-*Candida* agents, and their further testing is warranted as novel anti-*Candida* therapies [126].

CONCLUSIONS

Candida biofilms are intrinsically resistance to most antifungal drugs. However, the lipid formulations of amphotericin B and the echinocandins, used alone or in combination with the polyenes, do have activity against *Candida* biofilms. As an alternative strategy, antimicrobial lock therapy (ALT) has been proposed for the prevention and treatment of catheter-related bloodstream infections, often associated with biofilm formation. In this respect, liposomal amphotericin B lock therapy is an effective approach for treating *C. albicans* catheter-associated biofilms in a short treatment period. However, the development of new anti-biofilm agents is urgently needed since the number of therapeutic options for *Candida* biofilm-related diseases is very small. Furthermore, there is an increasing awareness of the hazards that are associated with the use of antibiotic and chemical agents. This has led to accelerated investigations on natural products, plants and their extracts as new sources of antimicrobial agents. The exploration of new and effective natural compounds with antifungal activity against *C. albicans* biofilm cells and with low cytotoxicity is likely to have a significant impact on the treatment and management of biofilm-associated fungal infections.

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