Role of biofilm in Candida infection
Many fungal and bacterial pathogens thrive as biofilms, causing infections in a variety of clinical and environmental niches [1,2]. As a biofilm, a microbial population demonstrates properties distinct from non-biofilm, free-floating cells. These cohesive communities of organisms are adherent to a surface and surrounded by an extracellular, polymeric matrix. They resist host defenses and may be extraordinarily tolerant to antimicrobials, withstanding anti-infectives at concentrations many times greater than those needed to eradicate non-biofilm cells [13]. The importance of biofilms has been increasingly evident with the widespread use of medical devices, as nearly all device-associated infections involve the biofilm mode of growth [24]. Vulnerable devices are diverse and include catheters, implants, pacemakers, artificial heart valves and CNS shunts [25].

Infection by Candida, the most common hospital-acquired fungal pathogen, frequently involves biofilm growth [5,6]. This organism has a propensity to adhere to the surface of catheters and other commonly used medical devices (Figure 1). In the hospital setting where medical device use is commonplace, Candida is the fourth most common cause of bloodstream infection and the third most common cause of urinary tract infection [7–10]. Exhibiting increased resistance to available antifungal therapies, these infections are notoriously difficult to treat. Most often, cure of biofilm infections requires removal of the infected device [11]. This is reflected in current guidelines that recommend removal of Candida-infected medical devices as treatment of the infection [12]. However, procedural risk to the patient may be significant, especially in the setting of permanent devices, such as pacemakers or hemodialysis access devices.

Efficacy of available antifungal therapies
The majority of medically important Candida spp. have now been shown to generate biofilms, including C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. tropicalis and C. parapsilosis [6,13]. Candida biofilms exhibit resistance to agents from all available, commonly used antifungal drug classes, including the azoles (fluconazole, itraconazole, voriconazole, posaconazole), the echinocandins (caspofungin, micafungin, anidulafungin), the amphotericin B formulations and flucytosine [14–19]. These antifungal drugs have diverse mechanisms of action. The azole drugs and amphotericin B formulations interfere with cell membrane ergosterol, while the echinocandins inhibit cell wall β-1,3 glucan synthesis and flucytosine, a pyrimidine analog, inhibits nucleic acid synthesis. [20]. The biofilm-associated drug-resistant phenotype has been replicated and studied using numerous in vitro models of biofilm infections [11,12,21–23]. In vivo
planktonic MICs shown to be active at concentrations 2- to 25-fold above their and agents in the echinocandin drug class, which have been against biofilms are the liposomal formulation of amphotericin B

Mechanisms of Candida biofilm resistance

Identification of the mechanisms underlying the resistance of Candida biofilms to antifungals has been of great interest [18,31–36]. Studies to date support a model in which multiple factors collectively promote resistance to anti-infectives during biofilm growth. There appears to be interplay between several resistance mechanisms, which vary during the phases of biofilm growth. Also, there is diversity among the biofilm cells in these heterogeneous structures. For example, subpopulations of Candida within biofilms are highly resistant to amphotericin B [33,34]. Upon drug treatment, these ‘persisters’ are proposed to act as a reservoir, allowing the biofilm to repopulate. An example of a phase-specific mechanism of resistance is the alteration of efflux pump activity. Upon transitioning to the biofilm lifestyle, the activity of C. albicans efflux pumps increases, ultimately lowering the intracellular azole drug concentrations and promoting resistance [18,31]. The contribution of the mechanism appears to be greatest early in biofilm formation, likely due to the lower cell density [37]. Other mechanisms are more specific to mature biofilm formation. For example, later in biofilm development, the increased cell density and extracellular matrix promotes resistance to amphotericin B, azoles and echinocandins [35–40]. In addition, the altered ergosterol content found in mature biofilm cells is hypothesized to influence drug resistance during the later growth phase [18].

The presence of an adhesive extracellular matrix, one of the unique and defining biofilm traits, has been linked to the multidrug-resistant phenotype observed for biofilms formed by many Candida spp., including C. albicans, C. parapsilosis, C. tropicalis, C. glabrata and C. krusei [3,35,36,38–40]. This mechanism has been shown to play a key role in biofilm resistance to amphotericin B, echinocandin drugs, fluconazole and fluconazole [40–43]. Several components of the extracellular matrix have been linked to biofilm resistance. The β-1,3 glucan content of the biofilm matrix correlates the antifungal-resistant phenotype and this substance has been shown to be a key factor in the binding or sequestering of both fluconazole and amphotericin B [43,44]. More recently, the importance of C. albicans eDNA has been recognized. Interestingly, this substance is critical for biofilm resistance to echinocandins and amphotericin B, but does not appear to contribute greatly toazole resistance [42]. While the importance of the biofilm matrix in biofilm drug resistance has become increasingly appreciated, how the individual components cooperate to produce this characteristic remains unclear, and may vary for the individual drugs.

As Candida transitions to the biofilm lifestyle, cellular stress responses become activated. These pathways contribute to the ability of Candida biofilms to resist environmental insults, including antifungal therapy. Pathways shown to contribute to azole resistance in Candida biofilms involve the mitogen-activated protein kinase, calcineurin and heat shock protein 90 (Hsp90p) [32,45–47]. These resistance pathways have been of great interest, as there are pharmaceutical agents that target calcineurin and Hsp90, which will be discussed below [45,47,48].

Strategies to combat Candida biofilms infections

As the available antifungal agents have minimal activity against biofilm infections, innovative approaches have been undertaken
to eradicate these infections (Figure 2). One of the strategies to optimize the efficacy of antifungals is delivery of high drug concentrations, most often by direct administration, such as lock therapy for an infected catheter [26,49–51]. Other investigations have examined the impact of targeting a variety of pathways involved in biofilm growth, including the stress responses, quorum sensing, extracellular matrix production and prostaglandin synthesis [45,47,48,52–54]. Disruption of these processes can greatly enhance the activity of antifungals during combination therapy. Lastly, screens of pharmaceutical and natural products have yielded promising compounds with excellent anti-biofilm activity [55–57].

High-dose therapy with available antifungals

While biofilms are capable of withstanding antifungals at the concentrations safely achievable in patients, they are susceptible to higher concentrations of a subset of these drugs, including amphotericin B and the echinocandins [28]. The toxicity of amphotericin B formulations limits their use at higher doses. Although azoles are relatively well-tolerated, Candida biofilms are tolerant to exceedingly high concentrations of these drugs [40–43]. Of the available antifungals, the echinocandins appear to have the most utility for biofilm treatment when administered systemically. Clinically, higher doses of these drugs have been used for treatment of biofilm infection, such as endocarditis [12]. However, even with the higher doses, biofilms often persist and physical removal of biofilm is ultimately required for cure [11,12].

Anti-infective lock therapy

Lock therapy, the prolonged instillation of drug into the catheter lumen, allows delivery of high-dose therapy while avoiding systemic toxicity. By directly administering drug to the infected site, local antifungal concentrations may safely reach 1000-fold higher than those achieved with systemic therapy [51]. Based on in vitro models, the antifungals anticipated to have the greatest anti-biofilm activity when administered in lock therapy are drugs in the echinocandin class and two amphotericin B formulations, liposomal amphotericin B and amphotericin B lipid complex [28,58]. In animal vascular catheter infection models, lock therapies with high doses of these agents (liposomal amphotericin B 10 mg/ml, liposomal amphotericin B and amphotericin B lipid complex 5 mg/ml and caspofungin 6.67 mg/ml) successfully eradicated C. albicans biofilms [26,49–51]. However, consistent with in vitro data, azole therapy (fluconazole 10 mg/ml) and low-dose echinocandin (0.25 μg/ml) were much less effective as lock therapy for treatment of the animal catheter infections [26,59]. Hudson et al. described an innovative amphotericin B formulation, a dextran-aldehyde-amphotericin B conjugate [60]. When mixed with carboxymethylcellulose-hydrazide, this compound forms a fungicidal gel. The product was well-tolerated in animals and has potential for future diverse uses in the local treatment of Candida biofilm infection. Possible applications include the treatment of vascular catheter, bone, joint and abdominal infections involving Candida biofilms.

No controlled trials have examined the efficacy of antifungal lock therapy for patients with fungal biofilm infections and data are limited to few case reports [51]. Lock therapy regimens used in patients primarily have included high doses of amphotericin B formulations or ethanol. Although several case reports describe high salvage rates, there is a concern for the possibility of publication bias. A randomized controlled trial is needed to determine if the lock therapies are effective, and if so, for which patient population. As lock therapy is delivered to the luminal surface only, biofilm infections involving the catheter tip or outer surface would likely have a higher failure rate. Also, if there is a concern for disseminated infection, concomitant systemic antifungal therapy may be required. In addition, there is the possibility that the use of an antifungal lock may promote development of resistance. Therefore, the use of antiseptics or non-antifungal agents is of interest. In vitro, EDTA, ethanol and high-dose minocycline (3 mg/ml) appear to be promising lock agents for treatment of Candida biofilms [61–63].

Exploiting combination therapy

One approach to increase the efficacy of antifungal therapies is to combine synergistic agents. This strategy has been shown to be successful for several drug classes for the treatment of Candida biofilms. Some of the most effective studies have included agents targeting stress responses activated during biofilm growth [45,47]. Uppuluri et al. identified calcineurin as a key regulator of C. albicans biofilm drug resistance [47]. The addition of inhibitors of calcineurin (cyclosporine A or tacrolimus) rendered in vitro biofilms susceptible to fluconazole. This synergy was recapitulated in vivo using combination lock therapy in a rat catheter biofilm infection model. Calcineurin inhibitors have since been shown to exhibit synergy with both an echinocandin (caspofungin) and amphotericin B [48]. The clinical availability of calcineurin inhibitors makes this combination therapy appealing. However, the immunosuppressive effects of these drugs
would likely preclude systemic administration and may limit their utility to lock therapy. Combination therapy also looks promising for agents targeting Hsp90p, a molecular chaperone governing Candida biofilm resistance [45]. Combination therapy with an Hsp90 inhibitor, geldanamycin, potentiated the activity of fluconazole against Candida biofilms.

The use of NSAIDs in combination with antifungal therapy shows potential for treatment of biofilm infections [52]. Medications in this drug class inhibit cyclooxygenases involved in the biosynthesis of mammalian prostaglandins. They are currently used for treatment of a variety of pain and inflammatory conditions. Interest for combination therapy arose from the discovery that several NSAIDs impair the ability of C. albicans to produce filaments and form biofilms in vitro [64]. One of the most potent inhibitors, aspirin, was even effective in eliminating established biofilms. This activity has been linked to inhibition of fungal prostaglandin E2 production [65]. Bink et al. explored the utility of NSAID-based combination therapy by testing the impact of combining an NSAID (diclofenac) with an echinocandin (caspofungin) [52]. In vitro, diclofenac acted synergistically with caspofungin and was most efficacious if administered during biofilm formation. In a subcutaneous device-associated biofilm model, treatment with diclofenac markedly enhanced the activity of caspofungin. This finding is exciting as NSAIDs are pharmaceutically available agents with established safety profiles. However, further studies are needed to determine the feasibility of this type of treatment regimen for patients. It is unclear if initiating the combination therapy in the setting of an established biofilm infection, as would be done clinically, would show this degree of efficacy.

To identify novel agents effective against C. albicans biofilms, LaFleur et al. screened compounds for synergistic activity of azoles [55]. They discovered that 2-adamantanamine, the structural analog of the antiviral amantadine, potentiated the activity of theazole, miconazole. The specific fungal target of 2-adamantanamine is unknown, but it is thought to inhibit a component of the ergosterol pathway upstream of the azole target, α-14 lanosterol demethylase. The safety profile of 2-adamantanamine has not been established. If the profile is similar to the antiviral amantadine, therapeutic systemic levels may be achievable [55].

Disrupting quorum sensing

As Candida cells proliferate, they communicate through quorum sensing, a process of sensing cell density [66]. One of these secreted molecules, farnesol, is an isoprenoid that inhibits the yeast-to-hyphae transition as well as the initiation of biofilm. At physiologic concentrations, this quorum sensing molecule does not appear to impact cells that have already begun to filament or biofilms that have become established [67]. However, at farnesol concentrations exceeding physiologic conditions, biofilms are degraded [68]. This observation suggests a potential use of farnesol, or a compound targeting this pathway, in the treatment of biofilm infections. In vitro data suggest that farnesol treatment may even enhance the activity of azoles [53,54]. In a non-biofilm murine candidiasis model, administration of farnesol was protective [69]. However, studies have not examined farnesol for treatment of biofilm infections in animal models. There is a concern that disruption of quorum sensing may provoke dispersion, the process of releasing cells from the biofilm, ultimately leading to disseminated disease [68]. Further studies of biofilms would be valuable to access the utility of targeting this quorum sensing pathway for Candida biofilm treatment.

Targeting extracellular matrix

One unique characteristic of biofilms, the presence of an extracellular matrix, contributes greatly to the antifungal resistance. The matrix elements specifically linked to this resistance, eDNA and β-1,3 glucan, are potential targets for anti-biofilm therapies [40–43]. When these components are disrupted by targeted enzymatic digestion, the efficacy of antifungals is greatly enhanced. For example, digestion of β-1,3 glucan potentiated fluconazole activity while degradation of eDNA enhanced amphotericin B activity in vitro [25,35,42]. The utility of targeted enzyme degradation would likely be limited to local therapy. An example that supports the feasibility of this approach is dornase alfa (Pulmozyme), a clinically available inhaled enzymatic treatment that targets the eDNA of bacterial biofilms in patients with cystic fibrosis and chronic pulmonary infections [70]. Another potential approach would be to identify inhibitors of the pathways known to regulate biofilm matrix production and delivery [44,71,72].

Natural products

Many natural products are active against biofilms formed by a variety of Candida spp. [73]. Compounds in investigation include peptides, oils, plant extracts and polyphenols from teas [56,57,74–80]. Although the results of these studies appear promising, the toxicities of many of the compounds have not been established and the tolerable dose in patients is unknown. One of the more well-studied compounds is carbohydrate-derived fulvic acid (CHD-FA), a heat stable, cationic, colloidal material [56]. CHD-FA is active against Candida biofilms as well as bacterial biofilms formed by oral bacteria that often accompany Candida in polymicrobial oral biofilm infections [56,81]. CHD-FA appears to non-specifically disrupt the fungal cell membrane and retains activity against many drug-resistant Candida strains. As it is relatively inexpensive and exhibits broad-spectrum anti-biofilm activity, it may be well suited for use as an oral antiseptic. The efficacy of CHD-FA has not been established in vivo, but the compound appears to be non-toxic in an epithelial cell line and a rat model [81].

To identify natural products with antifungal activity, Coleman et al. screened compounds using a high-throughput Caenorhabditis elegans assay [57]. This innovative approach concurrently assesses toxicity and treatment efficacy. With this screen, they identified 12 saponins with antifungal activity, several of which were active against C. albicans biofilms at concentrations without apparent toxicity. These compounds are proposed to target fungal ergosterol and form pores in the
Candida membrane. With a mechanism of action distinct from available drug classes, these compounds have the potential to have activity against resistant organisms or act synergistically with other antifungals.

Expert commentary & five-year view
It is becoming increasingly clear that Candida cells of a biofilm vary greatly from their free-floating counterparts. Therapies that inhibit planktonic Candida often have little impact on biofilms. Given the high prevalence of biofilm infection, the development of anti-Candida therapies should focus on this mode of growth. Models of Candida biofilms are critical for discovery of new antifungals and for investigating their efficacy. The development of high-throughput models is necessary for economically testing large libraries of molecules for anti-biofilm activity. One such model is the biofilm chip, which consists of nano-biofilms on a high-density microarray platform. A number of animal models with Candida biofilm infections closely mimicking patient infections will be of great value for examination of treatment efficacy.

To be the most useful medically, new antifungals would ideally be capable of completely eradicating a biofilm, as just a small nidus of infection may blossom to severe, recurrent infection. To achieve this degree of activity, several strategies may be considered for design of new drugs. One approach is to identify compounds with exquisite anti-Candida and anti-biofilm activity, effective against even the most resistant subpopulation of biofilm cells. Another approach is to uncover agents that disrupt biofilm processes, allowing traditional antifungals to attack biofilms when used on combination. Still another approach is to identify drugs that disrupt biofilm in a manner that allows the immune system to take hold, ultimately completely clearing the infection. As our understanding of Candida biofilms continues to grow, it will be fascinating to see how this information is applied to discovery of anti-biofilm drugs.

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Key issues
- Candida, one of the most common fungal pathogens, frequently grows as a biofilm adherent to a medical device or other surface.
- Candida biofilms exhibit increased resistance to antimicrobial therapies, including all available antifungal agents.
- The drug resistance of Candida biofilms is multifactorial. Contributing mechanisms include the presence of an extracellular matrix, increased activity of efflux pumps, increased cell density and induction of stress responses.
- Animal studies show that high doses of antifungals, such as liposomal amphotericin B and echinocandins, have anti-biofilm activity when delivered locally as catheter lock therapy.
- Therapies targeting the fungal stress responses (calcineurin, heat shock protein 90) can augment the action of antifungal drugs.
- Natural products, such as carbohydrate-derived fulvic acid and saponins, are promising compounds for treatment of Candida biofilms.
- A combination of high-throughput screens and animal models of Candida biofilm infection will be important for identifying and testing novel antifungals.

References


