Published in final edited form as:

Microbiol Spectr. 2015 June ; 3(3): . doi:10.1128/microbiolspec.MB-0007-2014.

From Biology to Drug Development: New Approaches to Combat the Threat of Fungal Biofilms

Christopher G. Pierce^{1,3}, Anand Srinivasan^{2,3}, Anand K. Ramasubramanian^{2,3}, and José L. López-Ribot^{1,3,*}

¹Department of Biology, The University of Texas at San Antonio

²Department of Biomedical Engineering, The University of Texas at San Antonio

³South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio

Abstract

Fungal infections constitute a major threat to an escalating number of critically ill patients. Fungi are eukaryotic organisms and, as such, there is a limited armamentarium of antifungal drugs, leading to high mortality rates. Moreover, fungal infections are often associated with the formation of biofilms, which contribute to virulence and further complicate treatment due to the high level of antifungal drug resistance displayed by sessile cells within these microbial communities. Thus, the treatment of fungal infections associated with a biofilm aetiology represents a formidable and unmet clinical challenge. The increasing importance and awareness of fungal biofilms is reflected by the fact that this is now an area of very active research. Studies in the last decade have provided important insights into fungal biofilm biology, physiology and pathology, as well as into the molecular basis of biofilm resistance. Here we discuss how this accumulated knowledge may inform the development of new anti-biofilm strategies and therapeutics that are urgently needed.

INTRODUCTION

Advances in modern medicine are prolonging the lives of severely ill individuals; however, at the same time they are creating an expanding population of compromised patients at increased risk of suffering from invasive fungal infections (1). These include surgical, transplant, cancer, ICU and HIV-infected patients, as well as neonates. Use of broad spectrum antibiotics, parenteral nutrition, medical implant devices, immune-suppression as well as disruption of mucosal barriers due to surgery, chemotherapy and radiotherapy represent the most important predisposing factors for these infections. Unfortunately, the mortality rates associated with these fungal infections remain unacceptably high, which clearly points to the many limitations of current antifungal therapy, including the limited armamentarium of antifungal agents, their inherent toxicity, and the emergence of resistance (2-4). Fungi are eukaryotic organisms and there is a paucity of selective targets which can be exploited for antifungal drug development, while at the same time this is also the main reason for the elevated toxicity of some of the current agents (2, 3, 5). Only three classes of

*Corresponding author. One UTSA Circle, San Antonio, Texas 78249, USA Phone: 1 210 458 7022, Fax: 1 210 458 7023 and jose.lopezribot@utsa.edu.

antifungal agents - azoles, polyenes and echinocandins - constitute the mainstay of antifungal therapy for patients with life-threatening invasive fungal infections. Moreover, the antifungal drug pipeline is mostly dry and, with the exception of isavuconazole, no new agents are expected to reach the market any time soon (2).

Formation of biofilms by many pathogenic fungi further complicates treatment (4). Biofilms are attached and structured microbial communities surrounded by a protective exopolymeric matrix (6-8). Once formed, these biofilms can initiate or prolong infections by providing a safe haven from which cells can invade local tissue, seed new infection sites and resist eradication efforts, and also lead to failure of implanted medical devices (9, 10). For example, yeasts and in particular *Candida* spp. are now the third most common microorganisms associated with catheter-related blood stream infections (11). The net result is that fungal biofilm formation carries important negative clinical consequences, adversely impacting the health of an increasing number of at-risk patients, and with soaring economic implications to our health care system. Clearly there is an urgent and unmet need for the development of novel antifungal agents, in particular those targeting fungal biofilm formation.

To efficiently treat fungal biofilms we need to understand their biology, physiology and implications for pathogenesis. Below we briefly review our current understanding of fungal biofilm development and the mechanisms of antifungal drug resistance which are operative in biofilms. We will then discuss how this accumulated knowledge can be harnessed in order to develop new approaches, including biofilm-eradication strategies and development of new anti-biofilm drugs, for the prevention and therapy of these difficult-to-treat infections.

FUNGAL BIOFILM DEVELOPMENT

Most information on fungal biofilm formation and on the structural characteristics of fungal biofilms comes from *in vitro* models developed during the last couple of decades (6, 7). Although early models for fungal biofilm formation were cumbersome, more recently research on fungal biofilms has been greatly facilitated by the development of relatively simple methodologies, most notably the 96-well microtiter plate model for the formation of fungal biofilms (12, 13). *In vitro* models have the advantage that they are amenable to careful control and manipulation, as multiple parameters can be modified and examined at the same time. This has allowed for the implementation of powerful molecular techniques (*i.e.* genetics, genomics and proteomics) for the dissection of pathways regulating fungal biofilm formation, along with state of the art microscopy techniques for the direct visualization and characterization of the overall structure of fungal biofilms.

It is now well established that fungal biofilm development is a complex process that occurs through multiple developmental stages (14, 15). These include initial adhesion to a substrate (which can be either a biologic or an abiotic surface), followed by cell division and proliferation, and biofilm maturation. In general, mature fungal biofilms exhibit a complex three-dimensional architecture with extensive spatial heterogeneity, and are typically surrounded by a matrix of self-produced exopolymeric material that accumulates as the biofilms mature and also contributes to cohesion (16-18). This spatial arrangement likely

represents an ideal adaptation that allows for the uptake of essential nutrients, the excretion of toxic products, and the communication between cells and the environment, and it is also responsible for the protection of sessile cells within the biofilms against immune defense mechanisms (8). Ultimately cells can detach from biofilms, and this dispersal step represents the return to a planktonic mode of growth while subsequently allowing for the colonization of new surfaces at distal sites, thereby completing the biofilm life-cycle (19). Importantly, although most of this structural information on fungal biofilms has been gathered using *in vitro* models, different groups of investigators have reported similar architectural features for *in vivo* formed biofilms, thereby corroborating the validity of these observations (20-25).

Development of fungal biofilms occurs in response to distinct environmental cues and is controlled, at the molecular level, by complex regulatory networks (6, 14, 26, 27). In the case of *C. albicans*, the most frequent causative agent of fungal biofilm infections, its ability to form biofilms is intimately linked to filamentation, and this may also hold true for other filamentous fungi (7, 28). In addition, as in their bacterial counterparts, cell-cell communication (*i.e.* quorum sensing) plays a critical role during the biofilm mode of growth in fungi (29, 30).

MECHANISMS OF FUNGAL BIOFILM RESISTANCE

As mentioned before, one of the most salient hallmarks of fungal biofilms is their extreme resistance against most conventional antifungal agents, which carries profound clinical implications. Very early studies already documented that sessile cells within these biofilms were up to 1,000 times more resistant to the fungistatic azoles (*i.e.* fluconazole) as compared to their planktonic counterparts, and they also showed decreased susceptibility against the polyenes, such as amphotericin B and nystatin (16, 17). We note here that polyenes display a decent biofilm activity *in vitro*; but unfortunately they do so at supraphysiological concentrations which are generally considered unsafe because of toxicity issues (31). Subsequent *in vitro* studies demonstrated excellent activity of therapeutic concentrations of liposomal formulations of Amphotericin B and the echinocandins against *Candida* biofilms (31-33); although this new class of antifungal agents may not be universally active against biofilms formed by other fungal species, in particular *Aspergillus fumigatus* (7, 34).

The resistance displayed by fungal biofilms is multifactorial in nature and may include molecular mechanisms of resistance that are relevant to planktonic cells, but also those that are unique to the biofilm mode of growth. For three excellent recent and comprehensive reviews on this topic, including the detailed description of mechanisms of resistance please refer to references (34-36). A potential list of contributory mechanisms to fungal biofilm resistance includes the increased cellular density within the biofilm (*i.e.* “safety in numbers”) (37); the existence of subpopulations of “persister” cells that develop tolerance to antifungal agents (38); the activation of efflux pumps, which may occur physiologically as a means to facilitate the removal of waste products but may concomitantly result in increased efflux of antifungal compounds (39); changes in the membrane sterols of sessile cells (40); overall changes in the metabolic and physiological status of cells, including cellular stress responses mostly orchestrated by the Hsp90 molecular chaperone also with connections to the calcineurin pathway (41); and, perhaps most notoriously, the physical blockage and

overall protective effect exerted by the biofilm matrix, mostly due to drug binding by exopolymeric components and presence of extracellular DNA (42, 43). Of note, the fact that fungal biofilm resistance is multifactorial poses a substantial challenge to the development of novel strategies – targeting just one of the multiple mechanisms may not be enough to circumvent resistance.

DEVELOPMENT OF NOVEL STRATEGIES AND THERAPEUTICS TO COMBAT ANTIFUNGAL BIOFILMS AND TO OVERCOME BIOFILM ANTIFUNGAL DRUG RESISTANCE

By now, the readers are probably appreciative of the intrinsic difficulties in treating fungal biofilm infections. The increasing understanding of fungal biofilms and the complexity of resistance mechanisms highlight many of the obstacles and the Herculean challenge ahead of us. Indeed, this is one of the urgent and unmet needs in Medical Mycology and arguably represents one of the top priorities in the field of antifungal drug research and development, which clearly indicates the dire medical, but also economical, need for new antifungal agents and strategies. The main question remains how we can harness these insights from fungal biofilm biology and pathogenesis, along with knowledge gained during the clinical management of patients, for the development of new strategies aimed at the control of fungal biofilms in clinical settings. When developing new drug candidates, it is imperative that the biofilm phenotype be taken into consideration, and the antifungal activity of the drug should be tested not only against planktonic cultures of a given organism but also against biofilms. Ideally this will be accompanied by optimization of different parameters for the targeting of fungal cells within biofilms, such as mode of action, penetration, and pharmacodynamic/pharmacokinetic properties.

Obviously, prevention of biofilm formation should be considered as the optimal strategy against a potential fungal biofilm infection, since once fully established these mature biofilms are much more difficult to treat and eradicate. Prophylactic regimens could represent important weapons particularly against infections in implanted medical devices including catheters (9, 10). These preventive approaches will target fungal cells while they remain planktonic and therefore still susceptible to therapy, or during the early stages of development by inhibiting their biofilm-forming ability. Nevertheless, some strategies can also be devised for the treatment of fully established (mature) fungal biofilms.

Below we present and discuss several main strategies that are being the focus of current research on this topic, and can be further developed, hopefully in the near future, to accomplish these lofty goals.

Modified surfaces and antimicrobial coatings

A wide range of biomaterials used for the fabrication of biomedical-assist devices have been shown to support adhesion, colonization and subsequent biofilm formation by different pathogenic fungi (10). Thus, in order to prevent infections associated with biofilm formation on the surface of implanted devices used in clinical practice, there is an interest in the development of advanced biomaterials which do not support microbial (both fungal and

bacterial) adhesion and colonization, as well the development of antimicrobial coatings that can inhibit adhesion of fungal cells and also display direct inhibitory activity through the release of drug from the biomaterial. A recent study tested the effect of surface and serum on biofilm development by different *Candida* clinical isolates (44). The materials tested included polycarbonate, polystyrene, stainless steel, Teflon, polyvinyl chloride or hydroxyapatite. Results indicated that a serum conditioning film increases the adherence to both metallic and non-metallic materials, and that roughness and hydrophobicity can modulate *C. albicans* biofilm formation. Another study reported that surface modifications of polyetherurethane, polycarbonateurethane and poly(ethyleneterephthalate) influenced fungal biofilm formation *in vitro* (45).

Bachmann and colleagues described that impregnation with different concentrations of caspofungin displayed an inhibitory role on subsequent biofilm formation of *C. albicans* on polystyrene surfaces (18). More recently, a similar inhibitory effect was demonstrated for voriconazole, as surfaces previously coated with this new generation azole (which is not active against preformed biofilms) showed reduced formation of biofilms by different *Candida* spp. (46). Thin-film coating of polymethylmethacrylate incorporating different antifungals, including nystatin, amphotericin B and chlorhexidine, effectively inhibited *C. albicans* biofilm formation as a potential preventive therapy for denture stomatitis; importantly these novel formulations allowed for the slow-release of drugs over time (47). In another study pre-coating the surface of acrylic disks with chlorhexidine or histatin 5 (a naturally occurring antimicrobial peptide in saliva), but not defensins, significantly inhibited *C. albicans* biofilm formation (48). Silver-coated catheters have been used in the clinics to inhibit microbial growth, and Monteiro and colleagues recently demonstrated that silver nanoparticles exert activity against fungal biofilms (49, 50).

Although very few studies have focused exclusively on antifungal surfaces, a recent article provides a comprehensive review of biomaterial surfaces capable of resisting fungal attachment and biofilm formation, and in particular surface coatings prepared with antifungal agents irreversibly immobilized (covalently bound) to the biomaterial surface. These agents may include cationic compounds, low molecular weight antiseptics, antimicrobial peptides and polyenes. When tethered covalently onto a biomaterial surface, these agents are not released nor become fouled, and in theory should remain permanently active at high concentrations to continue to kill or inhibit microorganisms upon contact (51).

Catheter Lock therapy

The widespread use of indwelling catheters in modern medicine, particularly central venous and hemodialysis catheters, has greatly contributed to the increasing incidence of fungal bloodstream infections, in particular candidiasis which is the third most frequent intravascular catheter-related infection with the overall highest crude mortality (9-11, 52). These infections may arise at any time during hospitalization, and most frequently contamination may occur by: i) intraluminal colonization of the hub and lumen of the device, ii) extraluminal colonization of the catheter, which typically originates from either the own patient's skin microbiota or from medical personnel, iii) hematogenous seeding from a distal site of infection, for example from the gastrointestinal tract following cytotoxic

therapy, and iv) less commonly from contaminated infusates (11, 52). Unfortunately, once a biofilm is formed on these catheters, removal of the medical device is often required in order to eliminate the infection; however, this is not always feasible due to clinical considerations (53, 54). Thus, the use of antifungal lock solutions is receiving increasing interest as a strategy to prevent and treat these infections (54, 55). Lock therapy utilizes prolonged instillation of a solution containing very high (suprapharmacological) concentrations of drugs, either antifungals or antiseptics, or combinations of both, to try to sterilize the infected catheter. Some of the most promising antifungal lock therapy strategies include the use conventional antifungal agents (in particular amphotericin B and the echinocandins because of their activity against preformed biofilms), antibacterial antibiotics which may display antifungal effects at high concentrations (such as minocycline, doxycycline and rifampin), antimicrobial peptides, and antiseptics (most frequently ethanol)(54, 56, 57). Interestingly, *in vitro* and *in vivo* assays have demonstrated that the ethanol effect on *C. albicans* biofilm formation is also due to its biochemical inhibition of alcohol dehydrogenase (Adh1p), as the enzymatic activity of Adh1p contributes to the ability of *C. albicans* to form biofilms (58). Very often anti-occlusive agents such as heparin, citrate or EDTA are also used in these solutions together with the antimicrobial agents (54). Despite the growing evidence for the efficacy of antifungal lock therapy from both *in vitro* and *in vivo* models of catheter-related fungal infections, to date, there is little comparative and clinical data between the different potential antifungal lock therapy strategies to permit specific recommendations for its use in the clinical management of patients (54).

High throughput phenotypic screens for identification of small molecule compounds with biofilm inhibitory activity

In high throughput screening (HTS), large libraries of small molecules are tested in *in vitro* assays in order to identify those molecules that affect a biological process of interest. These phenotypic screens can be used to investigate complex biological phenomena, such as biofilm formation by pathogenic fungi, while at the same time yield promising leads for the development of new antifungal drugs (3). They differ significantly from molecularly targeted therapeutics in that they require no detailed structural information about the target molecule, but rather allow for the selection of bioactive compounds through sampling large numbers of small molecules present in chemical libraries or repositories. One main attribute of HTS techniques in the drug development arena is their “hunger for speed”.

Recognizing the power of HTS, different groups during the last decade have used this approach for the identification of inhibitors of fungal biofilms. LaFleur and colleagues screened over 120,000 small molecule compounds present in the NIH Molecular Libraries Small Molecule Repository, and identified compounds with activity against *C. albicans* biofilms, and also those which enhanced the anti-biofilm activity of clotrimazole (an azole that does not display anti-biofilm activity by itself). Several of the most potent clotrimazole potentiators contained a 1,3-benzothiazole scaffold (59). More recently, and using similar HTS techniques, this same group identified 2-adamantanamine (AC17) as a chemosensitizer and potentiator of another azole antifungal, miconazole (60). The Johnson group screened for inhibitors of the budded-to-hyphal-form transition in *C. albicans*; the identified inhibitors likely inhibit multiple signalling pathways involved in filamentation, many of

which also regulate biofilm formation (61, 62). They subsequently reported that three of these inhibitors, buhytrinA, ETYA and CGP-37157, were capable of inhibiting *C. albicans* biofilm formation, although the biofilm-inhibitory activity varied among different clinical isolates (62). A similar screen, but this time for inhibitors of *C. albicans* adhesion to plastic, resulted in the identification “filastatin” (63). A more in depth characterization of this compound indicated that besides adhesion it also inhibits the yeast to hyphae transition and biofilm formation, both *in vitro* and *in vivo*, in a nematode model and in a mouse model of vulvovaginal candidiasis (63). More recently Wong and colleagues screened over 50,000 compounds in search for inhibitors of the *C. albicans* yeast to hyphae transition. Further examination of the antifungal activity of initial hits led to the discovery of SM21, which displays potent fungicidal activity and was more effective against *Candida* spp. biofilms than most current antifungals (64). *In vivo*, SM21 showed efficacy in the murine model of hematogenously disseminated candidiasis and in a mouse model of oral candidiasis. Pierce *et al.* performed cell-based phenotypic screens using three different chemical libraries from the National Cancer Institute's Open Chemical Repository collection, and identified several compounds with inhibitory activity against *C. albicans* biofilm formation and/or filamentation (65). However, most of the identified inhibitors displayed unacceptable levels of toxicity, which may limit their potential as candidates for the development of new antifungal drugs.

Our group has recently developed a technique for the nano-scale culture of fungal biofilms on a chip format, which allows for ultra-high-throughput drug screening (66, 67). The chip (termed “CaBChip” for *Candida albicans* Biofilm Chip) consists of a standard microscope glass slide containing over 1,200 individual *C. albicans* biofilms (‘nano-biofilms’), each with a volume of approximately 30 nanoliters, encapsulated in a three dimensional hydrogel matrix to simulate an environment which is close to the natural physiology (Figure 1). Despite a close to 3,000-fold miniaturization over conventional biofilms formed on microtiter plates, these “nano-biofilms” display similar phenotypic characteristics, including structural feature and antifungal drug resistance. This platform is robotically-controlled and operates in nano-scale volume, thereby reducing manual labor, reagent costs and assay duration. Although initially developed for *C. albicans* biofilms, these techniques can be adapted to other biofilm-forming fungal species, as well as to polymicrobial biofilms.

Drug repurposing: in search for novel antifungal and anti-biofilm activity of “old” drugs

The development of an entirely new drug is a long, difficult and extremely expensive process, estimated at 15 years of research and an investment of over US\$1.5 billion. The overall success rate is extremely low and, furthermore, any new drug has to undergo a highly demanding approval process by the Food and Drug Administration (FDA). Thus, repositioning or re-evaluating already FDA-approved drugs for new indications is gaining traction as an alternative path to accelerated drug development. Repurposing old drugs as antifungal agents may decrease the time and effort in bringing drugs with novel anti-fungal activity from the bench to the bedside (68).

An early report by the Douglas group already demonstrated the inhibitory effects of acetylsalicylic acid (aspirin) and other nonsteroidal anti-inflammatory drugs (NSAID, all

cyclooxygenase inhibitors) on *C. albicans* biofilm formation (69). High aspirin concentrations were also inhibitory against biofilms produced by other non-*albicans* *Candida* spp. (70). More recently the NSAID flufenamic acid, which has been specifically used for the treatment of rheumatoid arthritis, was reported to display inhibitory activity against *C. albicans* biofilms both alone and in combination with other antifungal agents (71). Another recent study reported on the screening of the Prestwick Library in order to identify inhibitors of *C. albicans* biofilm formation (72). This library consists of more than 1,200 FDA-approved, off-patent drugs with wide range of functions and mechanisms of action, and with well characterized pharmacological and toxicological properties. Among the identified bioactive drugs there were several with no known- or previously reported antifungal activity. Perhaps the most promising of these was Auranofin, which is a gold-containing compound with anti-inflammatory properties which has been used to treat rheumatoid arthritis for over 25 years. Importantly, Auranofin was also active against pre-formed *C. albicans* biofilms (72).

Natural Products

Plants and other natural products have been used extensively in folk medicine and are generally considered as good choices for a wide variety of drugs. Therefore they constitute potential sources for new antifungal drugs. Following the development of easy and inexpensive microtiter plate-based models of fungal biofilm formation and antifungal susceptibility testing there has been an increasing interest in the examination of the anti-biofilm activity of a variety of natural products. Compounds tested to date are too many to list individually, and readers are referred to a recent comprehensive review on this topic (73). An example illustrating the potential of natural products as anti-biofilm agents was recently provided by Delattin and colleagues (74). This research group had previously identified a decapeptide produced by the model plant *Arabidopsis thaliana* in response to infection by pathogenic fungi. They went on to demonstrate that OSIP108 inhibits *C. albicans* biofilm formation, enhances the anti-biofilm activity of conventional antifungal agents, and displays no cytotoxicity against human cells (74). The mechanism of action of this decapeptide may be related to its ability to interfere with cell wall components and with the cell wall integrity pathway (74). A possibility also is to use some of these natural products as potentiators of conventional antifungals; for example, You et al. reported that shearinines produced by *Penicillium* spp. synergistically enhanced the activity of amphotericin B against biofilms formed by *C. albicans* clinical isolates (75). Chitosan, a polymer of chitin isolated from crustacean exoskeletons, damaged *C. albicans* and *C. neoformans* biofilms, both *in vitro* and *in vivo* (76, 77).

Modulation of Quorum Sensing

As mentioned before, fungal biofilms constitute a remarkable and highly regulated developmental program (15). When part of a multicellular consortium, cells shift their behavior when they sense that their population density has reached a threshold level, at which point they communicate through small signaling molecules (29). This process is referred to as “quorum sensing”. Thus, another possibility to control fungal biofilms is by interrupting or modulating quorum sensing mechanisms.

In *C. albicans* farnesol and tyrosol constitute two main quorum-sensing molecules that act inversely in the regulation of morphogenetic conversions which also play a very important role in biofilm development (30, 78-80). However, treatment with farnesol, which inhibits filamentation and biofilm formation *in vitro*, increased *C. albicans* virulence *in vivo* in the murine model of hematogenously disseminated candidiasis (81), perhaps due to its potential to promote dissemination to distal sites (19). Farnesol also acts as a modulator of efflux mediated by multidrug efflux pumps and synergizes with conventional antifungals (82). In addition to farnesol, culture supernatants of *C. albicans* contain a mixture of autoregulatory alcohols that include isoamyl alcohol, 2-phenylethanol, 1-dodecanol, and E-nerolidol. In *in vitro* tests these molecules, alone or in combination, are capable of inhibiting *C. albicans* morphogenetic transitions. Moreover, when injected to mice, a cocktail solution containing a mixture of these alcohols led to increased survival and reduced organ burdens (83, 84).

In nature fungi and bacterial coexists in a wide variety of environments, and polymicrobial (mixed bacterial-fungal) biofilms may be more frequent than single-species biofilms. Reciprocal influences between bacteria and fungi within these communities can have dramatic effects on survival, development and pathogenesis. It has been demonstrated that some bacteria secrete quorum sensing and other antifungal molecules. For example, the 3-oxo-C12-acyl homoserine lactone-signaling molecule produced by *Pseudomonas aeruginosa* represses *C. albicans* morphologic transitions (85). Also, *P. aeruginosa*-produced phenazines inhibit filamentation, intercellular adherence, and biofilm development in *C. albicans* (86, 87). *Aspergillus*/bacterial biofilms are also associated with respiratory infections, particularly in the context of cystic fibrosis (7). Mixed bacterial-fungal communities are also commonly found in the oral environment, and mutanobactins secreted by *Streptococcus mutans* are capable of inhibiting or killing *C. albicans* biofilms (88, 89). A better understanding of these fungal-bacterial interactions may also serve as the basis for the development of cost-effective, low-risk strategies to control fungal infections through the use of probiotic organisms.

Targeting the Biofilm Matrix

A unique biofilm property is the production of an exopolymeric material (extracellular matrix) which encapsulates and protects fungal biofilm cells and contributes to the structural integrity of biofilms and their interaction with the surrounding environment. Two major components of this exopolymeric material, β -glucan and extracellular DNA, promote biofilm resistance to multiple antifungals. Therefore some strategies could be devised to target the fungal biofilm matrix that could lead to increased penetration of antifungals and/or to disaggregation of biofilm cells. One such approach has been described recently involving the application of enzymes that degrade the biofilm matrix. Martins and colleagues described the presence of extracellular DNA (eDNA) as a component of the *C. albicans* biofilm exopolymeric material and demonstrated its role in biofilm maintenance and stability. Results of a subsequent series of experiments indicated improved *in vitro* efficacy of DNase treatment of mature biofilms when used in combination with conventional antifungals. In the case of *A. fumigatus* biofilms, treatment with alginate lyase, an enzyme degrading the polysaccharides present within the biofilm matrix enhanced the activity of amphotericin B formulations, most likely by facilitating the drugs to reach the depths of the

biofilm (90). Also, a series of elegant studies by the Andes and Mitchell groups have provided important insights into the pathway for matrix glucan delivery which is induced during the *C. albicans* biofilm mode of growth, including the identification of key regulators of matrix biosynthesis and the characterization of several glucan-modification enzymes, which could represent additional targets for the development of promising anti-biofilm therapeutics (43, 91, 92).

Antibodies

Antibodies against certain adhesins can inhibit the initial attachment of fungal cells and impede subsequent biofilm development (93). A polyclonal anti-*C. albicans* antibody produced in chicken egg yolk prevented the adherence and biofilm formation by multiple *Candida* species (94). Antibody blocking of the *C. albicans* complement receptor 3-related protein significantly reduced adhesion and subsequent biofilm formation (95). Martinez and colleagues studied the effect of combinations of conventional antifungals and specific monoclonal antibodies against *C. neoformans* biofilms; however, the antibodies displayed a mostly antagonistic effect in combination with the antifungal drugs (96, 97). Importantly, the identification of antibodies with protective effects against fungal biofilms may open new avenues for the development of adjunctive and vaccination strategies for biofilm-associated infections, and area of increasing interest.

Combination therapy

The existence of different classes of antifungal drugs with different molecular targets opened new possibilities for combination therapy against fungal biofilms (4). However, an antagonistic trend has been described for combinations of azoles and echinocandins against biofilms, both when used concomitantly and in a sequential therapy regimen (azole first followed by an echinocandin) (98, 99).

Although biofilms formed by multiple clinical isolates of *C. albicans* displayed increased resistance to caspofungin after fluconazole pretreatment, the effect was not manifested by other non-*albicans Candida* spp. including *C. glabrata*, *C. dubliniensis* and *C. parapsilosis* (99).

As mentioned before, studies have implicated the molecular chaperone Hsp90 and the calcineurin pathway as key regulators of drug resistance in fungal biofilms (41, 100). Uppuluri et al. demonstrated that *C. albicans* cells in biofilms were resistant to individually delivered fluconazole or calcineurin inhibitors (cyclosporine, FK506 - tacrolimus), but exquisitely susceptible when the azole and the calcineurin inhibitor were used in combination (100). Moreover, combinatorial screening analysis demonstrated that different classes of antifungals that do not show activity against fungal biofilms can be turned effective in synergistic combination with relatively small doses of FK506 (66). Genetic or pharmacologic (with geldanamycin) depletion of Hsp90 reduced *C. albicans* biofilm growth and maturation, reduced glucan levels in the biofilm matrix, and abrogated resistance of *C. albicans* biofilms to the azole antifungal agents both *in vitro* and *in vivo*. Moreover, treatment with the Hsp90 inhibitor geldanamycin enhanced the efficacy of azoles and echinocandins against *Aspergillus fumigatus* biofilms *in vitro*. Thus, it would seem that

targeting Hsp90 may provide a potential strategy for the treatment of biofilm-associated infections caused by different fungal species.

Photodynamic therapy

Photodynamic therapy (PDT) represents a relatively new therapeutic technique for the treatment of a variety of diseases, including infectious diseases. The cytotoxic effects of PDT are generally mediated by the action of reactive oxygen species generated by the activation of a photosensitizer (dye) by light. PDT has potential applications for the treatment of fungal biofilm infections, in particular superficial mycoses such as denture stomatitis and oropharyngeal candidiasis (101-103). For example, when activated by a blue light emitting diode, the organic dye curcumin led to inactivation of both planktonic and sessile *C. albicans* cells (104, 105). Erythrosine activated by a green light reduced adherence to buccal epithelial cells and displayed significant antifungal effects against *C. albicans* biofilms formed *in vivo* in a murine model of oral candidiasis Erythrosine. Gold nanoparticle-enhanced photodynamic therapy of methylene blue demonstrated efficacy against otherwise recalcitrant biofilms formed by *C. albicans* (106).

CONCLUSIONS AND OUTLOOK

There is a desperate need for new antifungals, in particular those with anti-biofilm activity. Fungal biofilms are now an area of intensive research, with hundreds of groups around the globe working on this field, including both clinical and molecular aspects of fungal biofilm infections. Insights provided by these studies should guide the development of novel strategies to combat the increasing threat of these infections and to overcome fungal biofilm resistance. This will likely require a multi-disciplinary effort by clinicians and basic researchers, also with participation of governmental agencies as well as pharmaceutical and biotechnology companies. Successful development of these anti-biofilm strategies should have a profound impact on the management of patients suffering from these devastating fungal infections, potentially saving the lives of thousands of patients and significantly reducing the expenses to our health care system.

ACKNOWLEDGEMENTS

Biofilm work in the laboratory is funded by Grant numbered 1R01DE023510 from the National Institute of Dental & Craniofacial Research (to JLL-R), and by the Army Research Office of the Department of Defense under Contract No. W911NF-11-1-0136. Infectious diseases-related work in the A.K.R. laboratory is funded by NIH (SC1HL112629). CGP and AS acknowledge the receipt of predoctoral fellowships from American Heart Association, numbered 51PRE30004 and 13PRE17110093. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript, and the content is solely the responsibility of the authors.

REFERENCES

1. Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. Hidden killers: human fungal infections. *Sci Transl Med*. 2012; 4:165rv113.
2. Ostrosky-Zeichner L, Casadevall A, Galgiani JN, Odds FC, Rex JH. An insight into the antifungal pipeline: selected new molecules and beyond. *Nat Rev Drug Discov*. 2010; 9:719-727. [PubMed: 20725094]

3. Pierce CG, Lopez-Ribot JL. Candidiasis drug discovery and development: new approaches targeting virulence for discovering and identifying new drugs. *Expert opinion on drug discovery*. 2013; 8:1117–1126. [PubMed: 23738751]
4. Pierce CG, Srinivasan A, Uppuluri P, Ramasubramanian AK, Lopez-Ribot JL. Antifungal therapy with an emphasis on biofilms. *Curr Opin Pharmacol*. 2013; 13:726–730. [PubMed: 24011516]
5. Odds FC, Brown AJ, Gow NA. Antifungal agents: mechanisms of action. *Trends Microbiol*. 2003; 11:272–279. [PubMed: 12823944]
6. Fanning S, Mitchell AP. Fungal biofilms. *PLoS Pathog*. 2012; 8:e1002585. [PubMed: 22496639]
7. Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J. Our current understanding of fungal biofilms. *Crit Rev Microbiol*. 2009; 35:340–355. [PubMed: 19863383]
8. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL. *Candida* biofilms: an update. *Eukaryot Cell*. 2005; 4:633–638. [PubMed: 15821123]
9. Kojic EM, Darouiche RO. *Candida* infections of medical devices. *Clin Microbiol Rev*. 2004; 17:255–267. [PubMed: 15084500]
10. Ramage G, Martinez JP, Lopez-Ribot JL. *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res*. 2006; 6:979–986. [PubMed: 17042747]
11. Crump JA, Collignon PJ. Intravascular catheter-associated infections. *Eur J Clin Microbiol Infect Dis*. 2000; 19:1–8. [PubMed: 10706172]
12. Pierce CG, Uppuluri P, Tristan AR, Wormley FL Jr, Mowat E, Ramage G, Lopez-Ribot JL. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat Protoc*. 2008; 3:1494–1500. [PubMed: 18772877]
13. Ramage G, Vande Walle K, Wickes BL, Lopez-Ribot JL. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother*. 2001; 45:2475–2479. [PubMed: 11502517]
14. Blankenship JR, Mitchell AP. How to build a biofilm: a fungal perspective. *Curr Opin Microbiol*. 2006; 9:588–594. [PubMed: 17055772]
15. Nett J, Andes D. *Candida albicans* biofilm development, modeling a host-pathogen interaction. *Curr Opin Microbiol*. 2006; 9:340–345. [PubMed: 16815078]
16. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol*. 2001; 183:5385–5394. [PubMed: 11514524]
17. Ramage G, Vandewalle K, Wickes BL, Lopez-Ribot JL. Characteristics of biofilm formation by *Candida albicans*. *Rev Iberoam Micol*. 2001; 18:163–170. [PubMed: 15496122]
18. Bachmann SP, Patterson TF, Lopez-Ribot JL. In vitro activity of caspofungin (MK-0991) against *Candida albicans* clinical isolates displaying different mechanisms of azole resistance. *J Clin Microbiol*. 2002; 40:2228–2230. [PubMed: 12037093]
19. Uppuluri P, Chaturvedi AK, Srinivasan A, Banerjee M, Ramasubramanian AK, Kohler JR, Kadosh D, Lopez-Ribot JL. Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. *PLoS Pathog*. 2010; 6:e1000828. [PubMed: 20360962]
20. Andes D, Nett J, Oschel P, Albrecht R, Marchillo K, Pitula A. Development and characterization of an in vivo central venous catheter *Candida albicans* biofilm model. *Infect Immun*. 2004; 72:6023–6031. [PubMed: 15385506]
21. Kucharikova S, Tournu H, Holtappels M, Van Dijck P, Lagrou K. In vivo efficacy of anidulafungin against mature *Candida albicans* biofilms in a novel rat model of catheter-associated candidiasis. *Antimicrob Agents Chemother*. 2010; 54:4474–4475. [PubMed: 20660685]
22. Kucharikova S, Tournu H, Lagrou K, Van Dijck P, Bujdakova H. Detailed comparison of *Candida albicans* and *Candida glabrata* biofilms under different conditions and their susceptibility to caspofungin and anidulafungin. *J Med Microbiol*. 2011; 60:1261–1269. [PubMed: 21566087]
23. Lazzell AL, Chaturvedi AK, Pierce CG, Prasad D, Uppuluri P, Lopez-Ribot JL. Treatment and prevention of *Candida albicans* biofilms with caspofungin in a novel central venous catheter murine model of candidiasis. *J Antimicrob Chemother*. 2009; 64:567–570. [PubMed: 19584104]

24. Schinabeck MK, Long LA, Hossain MA, Chandra J, Mukherjee PK, Mohamed S, Ghannoum MA. Rabbit model of *Candida albicans* biofilm infection: liposomal amphotericin B antifungal lock therapy. *Antimicrob Agents Chemother*. 2004; 48:1727–1732. [PubMed: 15105127]
25. Shuford JA, Rouse MS, Piper KE, Steckelberg JM, Patel R. Evaluation of caspofungin and amphotericin B deoxycholate against *Candida albicans* biofilms in an experimental intravascular catheter infection model. *J Infect Dis*. 2006; 194:710–713. [PubMed: 16897672]
26. Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD, Tuch BB, Andes DR, Johnson AD. A recently evolved transcriptional network controls biofilm development in *Candida albicans*. *Cell*. 2012; 148:126–138. [PubMed: 22265407]
27. Nobile CJ, Mitchell AP. Genetics and genomics of *Candida albicans* biofilm formation. *Cellular microbiology*. 2006; 8:1382–1391. [PubMed: 16848788]
28. Lopez-Ribot JL. *Candida albicans* biofilms: more than filamentation. *Curr Biol*. 2005; 15:R453–455. [PubMed: 15964263]
29. Hogan DA. Talking to themselves: autoregulation and quorum sensing in fungi. *Eukaryot Cell*. 2006; 5:613–619. [PubMed: 16607008]
30. Ramage G, Saville SP, Wickes BL, Lopez-Ribot JL. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol*. 2002; 68:5459–5463. [PubMed: 12406738]
31. Ramage G, VandeWalle K, Bachmann SP, Wickes BL, Lopez-Ribot JL. In vitro pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. *Antimicrob Agents Chemother*. 2002; 46:3634–3636. [PubMed: 12384379]
32. Bachmann SP, VandeWalle K, Ramage G, Patterson TF, Wickes BL, Graybill JR, Lopez-Ribot JL. In vitro activity of caspofungin against *Candida albicans* biofilms. *Antimicrob Agents Chemother*. 2002; 46:3591–3596. [PubMed: 12384370]
33. Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob Agents Chemother*. 2002; 46:1773–1780. [PubMed: 12019089]
34. Ramage G, Rajendran R, Sherry L, Williams C. Fungal biofilm resistance. *Int J Microbiol*. 2012; 2012:528521. [PubMed: 22518145]
35. Mathe L, Van Dijck P. Recent insights into *Candida albicans* biofilm resistance mechanisms. *Current genetics*. 2013; 59:251–264. [PubMed: 23974350]
36. Taff HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of *Candida* biofilm drug resistance. *Future Microbiol*. 2013; 8:1325–1337. [PubMed: 24059922]
37. Perumal P, Mekala S, Chaffin WL. Role for cell density in antifungal drug resistance in *Candida albicans* biofilms. *Antimicrob Agents Chemother*. 2007; 51:2454–2463. [PubMed: 17502416]
38. LaFleur MD, Kumamoto CA, Lewis K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob Agents Chemother*. 2006; 50:3839–3846. [PubMed: 16923951]
39. Ramage G, Bachmann S, Patterson TF, Wickes BL, Lopez-Ribot JL. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J Antimicrob Chemother*. 2002; 49:973–980. [PubMed: 12039889]
40. Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect Immun*. 2003; 71:4333–4340. [PubMed: 12874310]
41. Robbins N, Uppuluri P, Nett J, Rajendran R, Ramage G, Lopez-Ribot JL, Andes D, Cowen LE. Hsp90 governs dispersion and drug resistance of fungal biofilms. *PLoS Pathog*. 2011; 7:e1002257. [PubMed: 21931556]
42. Martins M, Uppuluri P, Thomas DP, Cleary IA, Henriques M, Lopez-Ribot JL, Oliveira R. Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms. *Mycopathologia*. 2010; 169:323–331. [PubMed: 20012895]
43. Nett JE, Sanchez H, Cain MT, Andes DR. Genetic basis of *Candida* biofilm resistance due to drug-sequestering matrix glucan. *J Infect Dis*. 2010; 202:171–175. [PubMed: 20497051]
44. Frade JP, Arthington-Skaggs BA. Effect of serum and surface characteristics on *Candida albicans* biofilm formation. *Mycoses*. 2011; 54:e154–162. [PubMed: 20337936]

45. Chandra J, Patel JD, Li J, Zhou G, Mukherjee PK, McCormick TS, Anderson JM, Ghannoum MA. Modification of surface properties of biomaterials influences the ability of *Candida albicans* to form biofilms. *Appl Environ Microbiol*. 2005; 71:8795–8801. [PubMed: 16332875]
46. Valentin A, Canton E, Peman J, Martinez JP. Voriconazole inhibits biofilm formation in different species of the genus *Candida*. *J Antimicrob Chemother*. 2012; 67:2418–2423. [PubMed: 22733651]
47. Redding S, Bhatt B, Rawls HR, Siegel G, Scott K, Lopez-Ribot J. Inhibition of *Candida albicans* biofilm formation on denture material. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2009; 107:669–672. [PubMed: 19426921]
48. Pusateri CR, Monaco EA, Edgerton M. Sensitivity of *Candida albicans* biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. *Archives of oral biology*. 2009; 54:588–594. [PubMed: 19249746]
49. Monteiro DR, Silva S, Negri M, Gorup LF, de Camargo ER, Oliveira R, Barbosa DB, Henriques M. Antifungal activity of silver nanoparticles in combination with nystatin and chlorhexidine digluconate against *Candida albicans* and *Candida glabrata* biofilms. *Mycoses*. 2013; 56:672–680. [PubMed: 23773119]
50. Silva S, Pires P, Monteiro DR, Negri M, Gorup LF, Camargo ER, Barbosa DB, Oliveira R, Williams DW, Henriques M, Azeredo J. The effect of silver nanoparticles and nystatin on mixed biofilms of *Candida glabrata* and *Candida albicans* on acrylic. *Med Mycol*. 2013; 51:178–184. [PubMed: 22803822]
51. Coad BR, Kidd SE, Ellis DH, Griesser HJ. Biomaterials surfaces capable of resisting fungal attachment and biofilm formation. *Biotechnology advances*. 2014; 32:296–307. [PubMed: 24211473]
52. Raad I. Intravascular-catheter-related infections. *Lancet*. 1998; 351:893–898. [PubMed: 9525387]
53. Pappas PG, Kauffman CA, Andes D, Benjamin DK Jr, Calandra TF, Edwards JE Jr, Filler SG, Fisher JF, Kullberg BJ, Ostrosky-Zeichner L, Reboli AC, Rex JH, Walsh TJ, Sobel JD. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2009; 48:503–535. [PubMed: 19191635]
54. Walraven CJ, Lee SA. Antifungal lock therapy. *Antimicrob Agents Chemother*. 2013; 57:1–8. [PubMed: 23070153]
55. Cateau E, Rodier MH, Imbert C. In vitro efficacies of caspofungin or micafungin catheter lock solutions on *Candida albicans* biofilm growth. *J Antimicrob Chemother*. 2008; 62:153–155. [PubMed: 18407917]
56. Sherertz RJ, Boger MS, Collins CA, Mason L, Raad. Comparative in vitro efficacies of various catheter lock solutions. *Antimicrob Agents Chemother*. 2006; 50:1865–1868. [PubMed: 16641463]
57. Sousa C, Henriques M, Oliveira R. Mini-review: Antimicrobial central venous catheters--recent advances and strategies. *Biofouling*. 2011; 27:609–620. [PubMed: 21718230]
58. Mukherjee PK, Mohamed S, Chandra J, Kuhn D, Liu S, Antar OS, Munyon R, Mitchell AP, Andes D, Chance MR, Rouabhi M, Ghannoum MA. Alcohol dehydrogenase restricts the ability of the pathogen *Candida albicans* to form a biofilm on catheter surfaces through an ethanol-based mechanism. *Infect Immun*. 2006; 74:3804–3816. [PubMed: 16790752]
59. LaFleur MD, Lucumi E, Napper AD, Diamond SL, Lewis K. Novel high-throughput screen against *Candida albicans* identifies antifungal potentiators and agents effective against biofilms. *J Antimicrob Chemother*. 2011; 66:820–826. [PubMed: 21393183]
60. LaFleur MD, Sun L, Lister I, Keating J, Nantel A, Long L, Ghannoum M, North J, Lee RE, Coleman K, Dahl T, Lewis K. Potentiation of azole antifungals by 2-adamantanamine. *Antimicrob Agents Chemother*. 2013; 57:3585–3592. [PubMed: 23689724]
61. Midkiff J, Borochoff-Porte N, White D, Johnson DI. Small molecule inhibitors of the *Candida albicans* budded-to-hyphal transition act through multiple signaling pathways. *PLoS One*. 2011; 6:e25395. [PubMed: 21966518]
62. Grald A, Yargosz P, Case S, Shea K, Johnson DI. Small-molecule inhibitors of biofilm formation in laboratory and clinical isolates of *Candida albicans*. *J Med Microbiol*. 2012; 61:109–114. [PubMed: 21903824]

63. Fazly A, Jain C, Dehner AC, Issi L, Lilly EA, Ali A, Cao H, Fidel PL Jr, Rao RP, Kaufman PD. Chemical screening identifies filastatin, a small molecule inhibitor of *Candida albicans* adhesion, morphogenesis, and pathogenesis. *Proc Natl Acad Sci U S A*. 2013; 110:13594–13599. [PubMed: 23904484]
64. Wong SS, Kao RY, Yuen KY, Wang Y, Yang D, Samaranayake LP, Seneviratne CJ. In Vitro and In Vivo Activity of a Novel Antifungal Small Molecule against *Candida* Infections. *PLoS One*. 2014; 9:e85836. [PubMed: 24465737]
65. Pierce CG, Saville SP, Lopez-Ribot JL. High Content Phenotypic Screenings to Identify Inhibitors of *Candida albicans* Biofilm Formation and Filamentation. *Pathogens and disease*. 2014
66. Srinivasan A, Leung KP, Lopez-Ribot JL, Ramasubramanian AK. High-Throughput Nano-Biofilm Microarray for Antifungal Drug Discovery. *mBio*. 2013 E published ahead of print.
67. Srinivasan A, Uppuluri P, Lopez-Ribot J, Ramasubramanian AK. Development of a High-Throughput *Candida albicans* Biofilm Chip. *PLoS One*. 2011; 6:e19036. [PubMed: 21544190]
68. Tobinick EL. The value of drug repositioning in the current pharmaceutical market. *Drug news & perspectives*. 2009; 22:119–125. [PubMed: 19330170]
69. Alem MA, Douglas LJ. Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of *Candida albicans*. *Antimicrob Agents Chemother*. 2004; 48:41–47. [PubMed: 14693516]
70. Stepanovic S, Vukovic D, Jesic M, Ranin L. Influence of acetylsalicylic acid (aspirin) on biofilm production by *Candida* species. *J Chemother*. 2004; 16:134–138. [PubMed: 15216946]
71. Chavez-Dozal AA, Jahng M, Rane HS, Asare K, Kulkarny VV, Bernardo SM, Lee SA. In vitro analysis of flufenamic acid activity against *Candida albicans* biofilms. *Int J Antimicrob Agents*. 2014; 43:86–91. [PubMed: 24156913]
72. Siles SA, Srinivasan A, Pierce CG, Lopez-Ribot JL, Ramasubramanian AK. High-throughput Screening of a Collection of Known Pharmacologically Active Small Compounds for the Identification of *Candida albicans* Biofilm Inhibitors. *Antimicrob Agents Chemother*. 2013
73. Sardi JC, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJ. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J Med Microbiol*. 2013; 62:10–24. [PubMed: 23180477]
74. Delattin N, De Brucker K, Craik DJ, Cheneval O, Frohlich M, Veber M, Girandon L, Davis TR, Weeks AE, Kumamoto CA, Cos P, Coenye T, De Coninck B, Cammue BP, Thevissen K. The plant-derived decapeptide OSIP108 interferes with *Candida albicans* biofilm formation without affecting cell viability. *Antimicrob Agents Chemother*. 2014
75. You J, Du L, King JB, Hall BE, Cichewicz RH. Small-molecule suppressors of *Candida albicans* biofilm formation synergistically enhance the antifungal activity of amphotericin B against clinical *Candida* isolates. *ACS chemical biology*. 2013; 8:840–848. [PubMed: 23387427]
76. Martinez LR, Mihi MR, Han G, Frases S, Cordero RJ, Casadevall A, Friedman AJ, Friedman JM, Nosanchuk JD. The use of chitosan to damage *Cryptococcus neoformans* biofilms. *Biomaterials*. 2010; 31:669–679. [PubMed: 19819009]
77. Martinez LR, Mihi MR, Tar M, Cordero RJ, Han G, Friedman AJ, Friedman JM, Nosanchuk JD. Demonstration of antibiofilm and antifungal efficacy of chitosan against candidal biofilms, using an in vivo central venous catheter model. *J Infect Dis*. 2010; 201:1436–1440. [PubMed: 20331379]
78. Alem MA, Oteef MD, Flowers TH, Douglas LJ. Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. *Eukaryot Cell*. 2006; 5:1770–1779. [PubMed: 16980403]
79. Chen H, Fujita M, Feng Q, Clardy J, Fink GR. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc Natl Acad Sci U S A*. 2004; 101:5048–5052. [PubMed: 15051880]
80. Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, Dussault P, Nickerson KW. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol*. 2001; 67:2982–2992. [PubMed: 11425711]
81. Navarathna DH, Hornby JM, Krishnan N, Parkhurst A, Duhamel GE, Nickerson KW. Effect of farnesol on a mouse model of systemic candidiasis, determined by use of a DPP3 knockout mutant of *Candida albicans*. *Infect Immun*. 2007; 75:1609–1618. [PubMed: 17283095]

82. Sharma M, Prasad R. The quorum-sensing molecule farnesol is a modulator of drug efflux mediated by ABC multidrug transporters and synergizes with drugs in *Candida albicans*. *Antimicrob Agents Chemother*. 2011; 55:4834–4843. [PubMed: 21768514]
83. Martins M, Henriques M, Azeredo J, Rocha SM, Coimbra MA, Oliveira R. Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signaling molecules produced by planktonic and biofilm cells. *Eukaryot Cell*. 2007; 6:2429–2436. [PubMed: 17981993]
84. Martins M, Lazzell AL, Lopez-Ribot JL, Henriques M, Oliveira R. Effect of exogenous administration of *Candida albicans* autoregulatory alcohols in a murine model of hematogenously disseminated candidiasis. *J Basic Microbiol*. 2012; 52:487–491. [PubMed: 22052380]
85. Hogan DA, Vik A, Kolter R. A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Mol Microbiol*. 2004; 54:1212–1223. [PubMed: 15554963]
86. Gibson J, Sood A, Hogan DA. *Pseudomonas aeruginosa*-*Candida albicans* interactions: localization and fungal toxicity of a phenazine derivative. *Appl Environ Microbiol*. 2009; 75:504–513. [PubMed: 19011064]
87. Morales DK, Jacobs NJ, Rajamani S, Krishnamurthy M, Cubillos-Ruiz JR, Hogan DA. Antifungal mechanisms by which a novel *Pseudomonas aeruginosa* phenazine toxin kills *Candida albicans* in biofilms. *Mol Microbiol*. 2010; 78:1379–1392. [PubMed: 21143312]
88. Joyner PM, Liu J, Zhang Z, Merritt J, Qi F, Cichewicz RH. Mutanobactin A from the human oral pathogen *Streptococcus mutans* is a cross-kingdom regulator of the yeast-mycelium transition. *Org Biomol Chem*. 2010; 8:5486–5489. [PubMed: 20852771]
89. Wang X, Du L, You J, King JB, Cichewicz RH. Fungal biofilm inhibitors from a human oral microbiome-derived bacterium. *Org Biomol Chem*. 2012; 10:2044–2050. [PubMed: 22281750]
90. Bugli F, Posteraro B, Papi M, Torelli R, Maiorana A, Paroni Sterbini F, Posteraro P, Sanguinetti M, De Spirito M. In vitro interaction between alginate lyase and amphotericin B against *Aspergillus fumigatus* biofilm determined by different methods. *Antimicrob Agents Chemother*. 2013; 57:1275–1282. [PubMed: 23263007]
91. Nobile CJ, Nett JE, Hernday AD, Homann OR, Deneault JS, Nantel A, Andes DR, Johnson AD, Mitchell AP. Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol*. 2009; 7:e1000133. [PubMed: 19529758]
92. Taff HT, Nett JE, Zarnowski R, Ross KM, Sanchez H, Cain MT, Hamaker J, Mitchell AP, Andes DR. A *Candida* biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog*. 2012; 8:e1002848. [PubMed: 22876186]
93. Lopez-Ribot JL, Casanova M, Murgui A, Martinez JP. Antibody response to *Candida albicans* cell wall antigens. *FEMS Immunol Med Microbiol*. 2004; 41:187–196. [PubMed: 15196567]
94. Fujibayashi T, Nakamura M, Tominaga A, Satoh N, Kawarai T, Narisawa N, Shinozuka O, Watanabe H, Yamazaki T, Senpuku H. Effects of IgY against *Candida albicans* and *Candida* spp. Adherence and Biofilm Formation. *Japanese journal of infectious diseases*. 2009; 62:337–342. [PubMed: 19762981]
95. Bujdakova H, Paulovicova E, Paulovicova L, Simova Z. Participation of the *Candida albicans* surface antigen in adhesion, the first phase of biofilm development. *FEMS Immunol Med Microbiol*. 2010; 59:485–492. [PubMed: 20618846]
96. Martinez LR, Casadevall A. *Cryptococcus neoformans* biofilm formation depends on surface support and carbon source and reduces fungal cell susceptibility to heat, cold, and UV light. *Appl Environ Microbiol*. 2007; 73:4592–4601. [PubMed: 17513597]
97. Martinez LR, Christaki E, Casadevall A. Specific antibody to *Cryptococcus neoformans* glucuronoxylomannan antagonizes antifungal drug action against cryptococcal biofilms in vitro. *J Infect Dis*. 2006; 194:261–266. [PubMed: 16779734]
98. Bachmann SP, Ramage G, VandeWalle K, Patterson TF, Wickes BL, Lopez-Ribot JL. Antifungal combinations against *Candida albicans* biofilms in vitro. *Antimicrob Agents Chemother*. 2003; 47:3657–3659. [PubMed: 14576141]
99. Sarkar S, Uppuluri P, Pierce CG, Lopez-Ribot JL. In vitro study of sequential fluconazole and caspofungin treatment against *Candida albicans* biofilms. *Antimicrob Agents Chemother*. 2014; 58:1183–1186. [PubMed: 24217700]

100. Uppuluri P, Nett J, Heitman J, Andes D. Synergistic effect of calcineurin inhibitors and fluconazole against *Candida albicans* biofilms. *Antimicrob Agents Chemother*. 2008; 52:1127–1132. [PubMed: 18180354]
101. Costa AC, Pereira CA, Freire F, Junqueira JC, Jorge AO. Methods for obtaining reliable and reproducible results in studies of *Candida* biofilms formed in vitro. *Mycoses*. 2013
102. Junqueira JC, Jorge AO, Barbosa JO, Rossoni RD, Vilela SF, Costa AC, Primo FL, Goncalves JM, Tedesco AC, Suleiman JM. Photodynamic inactivation of biofilms formed by *Candida* spp., *Trichosporon mucoides*, and *Kodamaea ohmeri* by cationic nanoemulsion of zinc 2,9,16,23-tetrakis(phenylthio)-29H, 31H-phthalocyanine (ZnPc). *Lasers in medical science*. 2012; 27:1205–1212. [PubMed: 22278349]
103. Pereira CA, Romeiro RL, Costa AC, Machado AK, Junqueira JC, Jorge AO. Susceptibility of *Candida albicans*, *Staphylococcus aureus*, and *Streptococcus mutans* biofilms to photodynamic inactivation: an in vitro study. *Lasers in medical science*. 2011; 26:341–348. [PubMed: 21069408]
104. Dovigo LN, Pavarina AC, Carmello JC, Machado AL, Brunetti IL, Bagnato VS. Susceptibility of clinical isolates of *Candida* to photodynamic effects of curcumin. *Lasers in surgery and medicine*. 2011; 43:927–934. [PubMed: 22006736]
105. Dovigo LN, Pavarina AC, Ribeiro AP, Brunetti IL, Costa CA, Jacomassi DP, Bagnato VS, Kurachi C. Investigation of the photodynamic effects of curcumin against *Candida albicans*. *Photochemistry and photobiology*. 2011; 87:895–903. [PubMed: 21517888]
106. Khan S, Alam F, Azam A, Khan AU. Gold nanoparticles enhance methylene blue-induced photodynamic therapy: a novel therapeutic approach to inhibit *Candida albicans* biofilm. *International journal of nanomedicine*. 2012; 7:3245–3257. [PubMed: 22802686]

Figure 1. Images depicting the *CaBChip* as new technology platform for microbial culture at the nano-scale level. The technique allows for a variety of high-throughput applications, including ultra- high-throughput drug screening.