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Innovations in the field of fungal biofilms: looking for new targets and new chemical compounds

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SUMMARY

Biofilms pose a serious problem for public health. Penetration of pharmacological agents within a biofilm is hampered by the morphological structure of such microbial communities. A biofilm infection therefore entails adverse outcomes both in the field of cost management and patient prognosis. The problem is further complicated if the drugs available to combat a biofilm-related fungal infection versus a bacterial one are compared: in the case of a fungal infection, the drugs available are less efficacious than antibiotics used to counteract a bacterial infection. Furthermore, even the fairly recent introduction of antifungals, such as echinocandins, start presenting some limits of usage, such as ineffectiveness in treating some fungal populations and increased resistance. It therefore becomes imperative to search for innovative molecules in order to combat this condition. The discovery of new molecules and/or new targets can make a difference. This paper illustrates the main innovative molecules that are coming to light in the field of infection by fungal biofilms.

Keywords: fungal biofilm, *Candida* spp., infections, innovative molecules.

INTRODUCTION

In recent years, nosocomial infections are exponentially progressing, generating serious disadvantages [1]. These problems have an impact on the patient (increased mortality) and on the National Health Service Management (long-term care costs and increased consumption of antimicrobials).
A biofilm-associated infection, is more difficult to treat than a non associated one. This is due to the particular phenotype of biofilm that allows the survival of the microbes internally contained, against "external aggression", such as drugs [2].

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Candida and biofilms

Microbial biofilms represent real biological systems with a high degree of organization, whereas microbes are structured and coordinated into a functional community, capable of cooperating in the metabolic-reproductive and infectious processes. Several species of *Candida*, in particular *albicans, parapsilosis, glabrata,* and *tropicalis* develop biofilms on different types of devices [3, 4].
A *Candida* biofilm, in particular the one formed by *C. albicans*, which is the most frequently isolated fungal pathogen, includes two types of cells: small oval yeast-cells (blastospores) and the hyphae, elongated cells in tubule. The biofilms that grow *in vitro* often have a base of yeast-like cells from which emerges the layer of hyphae and pseudohyphes and in higher a yeast-cell layer which germinate from hyphae, immersed in an extracellular matrix [5]. The structure of the *in vivo Candida* biofilms (central venous catheter model) appears

more complex than that *in vitro*, with yeast-like cells and hyphae intersperse in the extracellular matrix [6]. Genetic studies indicate that both the yeast-like cells and the hypha are important in the formation of a fungal biofilm [7].
Candida biofilm formation *in vitro* is a microbiological process that involves several steps. In the process of joining the yeast-cells adhere to the substrate, in the initial stage proliferate and produce hyphae and pseudohyphae. In the maturation phase, it accumulates in the extracellular matrix material, increases the biomass of biofilm and its resistance against drugs.
Finally, in the separation and dispersion phases, the yeast-cells are released from the biofilm and colonize the surrounding environment. In reality, these steps do not occur sequentially, as previously described, but simultaneously *in vivo*.
Biofilm formation requires coordination, interaction and communication and is regulated by the exchange of intercellular chemical signals, known as "quorum-sensing." It is a complex system of communication between microorganisms able to respond to the growth of microbial population density through the transcriptional activation of genes, the synthesis of numerous substances and virulence factors.
Today the genes that regulate (positively or negatively) the different stages of fungal biofilm formation are known: many genes coded for cell wall proteins (Eap1, Hwp1, ALS1, Als3) and perform a key role in the cell-substrate adhesion phase or cell-cell interaction [5]. Some are involved in the hyphae (filamentation) production phase: for example in the quorum sensing of *Candida albicans* the farnesol is an inhibitor of the hyphae formation while the tyrosol has an opposite activity to that of the farnesol and stimulates their formation [8, 9]. Some genes code transcription factors and protein kinases (bcr1, Zap1) that act as regulators of the same biofilm structure for example by influencing the production of the extracellular matrix, which is composed of carbohydrates (glucans and mannans), proteins, hexosamine and extracellular DNA [10, 11].

Candidiasis
Candida albicans, a member of the *Candida* family, is the most common fungal pathogen responsible of candidiasis [12]. It acts as a commensal against the human organism colonizing mucosal surfaces, particularly those of the skin, gastrointestinal tract, oral cavity and the genitourinary tract. In immunocompetent patients it does not cause damage or it can damage the host but without serious consequences, living as a symbiont with them [13]. In immunocompromised patients, however, it can cause various types of infections, ranging from mild illnesses in the mucous membranes, to an invasive candidiasis that can threaten the patient's life itself [12].
The latter, with serious infections, occur frequently in hospitals, favoured by immunosuppressive therapy, the use of broad-spectrum antibiotics and the use of implantable medical devices or intravenous catheters [12].
From an analysis conducted in US hospitals, it was found that candidiasis is associated with a 14.5% increase in mortality, an increase in the average duration of hospital stay of 10.1 days and a rise of hospital expenditure amounted to $ 39.331 [14].

Conventional drug therapy
The antifungal drugs used against a *Candida* infection - such as azoles, polyenes and echinocandins -, are extremely limited especially when compared to the more nourished quantity of antibacterial drugs [15]. Unfortunately, each of these drug classes present some disadvantages: the polyenes nephrotoxicity, mitigated only in part by the liposomal formulations; azoles increased resistance and the inability to act toward some fungal species encased in biofilms [16].
Even echinocandins, antifungals which have been recently introduced, with an excellent safety profile, are not always able to fight a *Candida* infection, due to the continuous increase of species resistant to therapy [17, 18].
In the case of biofilms from *Candida* spp. the echinocandins (*e.g.* caspofungin) are antifungals that are the most active in the eradication of infection [19]. With a biofilm associated infection, the best practice is the removal of the infected implant or organ, but if the biofilm cannot be mechanically removed, it is necessary to use more complex patterns of antimicrobial therapy in terms of choice of the molecules, the administration and therapy times [20].

Resistance
The primary purpose of biofilm formation is the protection of the microorganisms from external

environmental aggressions, the metabolic cooperation and the resulting increase in resistance to antimicrobials.
Fungi associated in biofilms exhibit greater resistance, up to 1000 times compared to planktonic forms against antifungal agents and may express different mechanisms of resistance [3]. The slow growth by reduction of nutrients and / or chemical-physical changes in the biofilm is a physiological effectiveness limitation of amphotericin B and other antimycotics. The cell density within the biofilm is an important factor of resistance within the cell population formed by yeast-cells and hyphae and physically hinders the achievement of deeper layers of the antimycotic, in particular the azoles, at the same time allowing a cooperation between the various components through the "quorum sensing" molecules. The extracellular polysaccharide matrix acts as a "physical barrier" and/or "reactive barrier" and retards the diffusion of antimycotic or hinders the penetration through the biofilm (b-1,3 glucan in the biofilm matrix binds and captures the azoles) [5].
A greater resistance to the azoles and polyenes is given by the characteristics of the cells that constitute the mature biofilms that have a reduced level of membrane sterols and a high expression of ergosterol biosynthesis genes, perhaps in response to hypoxia [21, 22]. In addition, the CDR1, CDR2 MDR1 genes are early induced during biofilm formation, regulating membrane transporters and efflux pumps and may contribute to resistance to azoles [23]. In an elegant work, Sherry et al. have correlated the ability to form biofilms by different strains of *Candida* to their pathogenicity (morbidity and mortality) and gene transcription, identifying specific biofilm genes that are upregulated in *Candida* strains that produce many biofilms [24].
Finally, within the biofilm subpopulations with differentiated phenotype "persisters" cells are developed, which are stochastically formed, which are highly tolerant to drugs (*Candida albicans, Candida krusei, Candida parapsilosis* against amphotericin B) (physiological resistance) [3, 25]. It is phenotypic variants, not mutants, which are crucial in the persistence of infection and determinants in treatment failures [26].
The biofilm is a condition that favours the formation of "more virulent" microorganism reservoirs such as the herpes HSV-1 virus and Coxackievirus CVBS both in the environment and in healthy and sick carriers, hence the need to implement prevention strategies of biofilm formation and checks in subjects with infections risks related to medical devices (isolated capable of producing clinical outcome-predictive marker biofilms?) [24, 27, 28].

The new therapeutic challenges
Antibiotics and conventional antimycotics for their bactericidal or fungicidal, or, bacteriostatic or fungistatic mechanism of action, as a consequence, generate, the development of resistant strains to an antimicrobial treatment. This phenomenon is due to the strong selective pressure that is created in involved bacteria or fungi [29]. For this reason, it is important to discover antimicrobial agents with new mechanisms of action or research molecules which are capable of working in synergy with existing antimycotics, in order to generate a better therapeutic response.

The synergistic effect of a combined therapy
Fluconazole and terpenoids of plant origin
In a study published in 2014, Doke and colleagues evaluated the effectiveness of three biologically active terpenoids such as carvacrol, eugenol and thymol in combination with fluconazole against planktonic cells, the biofilm development and already mature *Candida albicans* biofilm [30]. In particular, the results indicate that biofilm development and mature biofilms of the species will show resistance to treatment with only azole. Combinations of carvacrol- fluconazole and eugenol- fluconazole, however, are shown to be effective against forming biofilm and the association of carvacrol-fluconazole inhibits mature biofilm [30].

Clotrimazole and molecules from the benzothiazolic skeleton
Candida albicans generates infections in the female genital tract and oral and pharyngeal candidiasis which is often difficult to treat, partly because the infection can recur. It is believed that this phenomenon may be due to the presence of biofilms [31, 32]. Such infections are treated with azole drugs, in particular with clotrimazole. The drug that has an effect on the α-14lanosterol demethylase resulting in the arrest of fungus cell growth, is not always able to fight the biofilm infection, given the continuous increase of species resistant to therapy [33].

LaFleur and colleagues, in a study published in 2011, have discovered several compounds which enhanced the clotrimazole's activity [33]. These chemical compounds increasing the activity of the drug from several points of view, precisely improving the dosing regimens, decreasing the concentration of the requested drug for the therapy and, not least, fighting the resistance [33]. Among the molecules that synergized with clotrimazole, the most powerful, showed a 1,3 benzothiazolic scaffold [33].
Benzothiazole components have antifungal activity, so these molecules can potentiate the antifungal activity of the azole [34].
Future studies will determine if the molecules with this chemical skeleton are able to inhibit the filamentation, a key factor in the development of a *Candida albicans* biofilm, a recognized mechanism of action for the 6-amino-2-*n*-pentylthiobenzothiazole compound or how to exercise their antifungal effect [35, 33].
Unfortunately, some of these molecules with a 1,3 benzotiazolic skeleton seems to be cytotoxic. It may be possible to optimize this scaffold for reduced cytotoxicity by synthesizing and testing chemical analogues of this moiety [33].

DNAse and antifungal drugs
The extracellular DNA (Edna), a component of the biofilm matrix, is one of the elements that guarantee the integrity of the microbial consortium [36]. Martins and colleagues, in a study published on Mycoses in 2012, wanted to test the DNase influence, or compounds capable of degrading the extracellular DNA, in combination with the main antifungal drugs, employable in the case of a *Candida albicans* biofilm [37]. Specifically, the following combinations were evaluated: DNase and amphotericin B, DNase and fluconazole, DNase and caspofungin. The results show that the most synergistic combination is that formed by the DNAse- amphotericin B: the DNAse or deoxyribonuclease I increase *C. albicans* biofilm cells susceptibility to this polyene drug [37]. Further studies will be needed in order to test the *in vivo* efficacy of DNase and to understand its exact mechanism of action [37].
It is reasonable to think, however, that the destruction of the biofilm integrity allows a better uptake of the drug by the fungal cell enclosed in its interior.

The research of new molecular mechanisms of action
The use of conventional antimicrobials, according to what has been previously described, further strengthens the development of resistant strains to pharmacological treatment. It is strictly actual therefore, to search for antimicrobials with the most updated mechanisms of action.
Among the perspectives that are emerging in the research field of infectious disease on biofilm, the most promising are those directed in attacking the virulence factors, elements responsible for the guest's damage [29,38]. Attacking this target offers indisputable advantages: enlarging the therapeutic targets, allowing the development of new antifungals which will safeguard the "useful microbes" of the human microbial flora, otherwise eliminated by a conventional antimicrobial treatment and lastly creating a minor drug resistance [29].

The contrast to the virulence factors in *Candida* spp.
The virulence factors of *Candida* spp. generating damage to the host consist in its ability to form a biofilm and the ability of the fungus to reach out to filamentation [39].
If the biofilm formation is avoided, consisting of microbiological steps mentioned above, is also lacking the biofilm associated infection and thus the serious damage this causes to the colonized host.
To prevent biofilm formation in the health field some rules must be observed, first of all, the hand washing, to prevent the germ's transmission and the use of medical devices associated with the lowest degree of microbial contamination [40]. This results in the choice of catheters with a lowest degree of microbial adhesion or in the use of devices covered by materials with antimicrobial properties, such as, for example, silver nanoparticles [41].
If the biofilm is in the developing phase, we should try to counteract it with several techniques, specific to each phase of biofilm's formation. The research is focusing on discovering, for example, specific molecules that act in the maturation's phase, such as farnesol for *Candida* spp.
The filamentation is the morphological transition process from a yeast form to that of hypha, supported by host factors such as a temperature of 37°C, the presence of serum, nutrients [39]. If yeasts have greater disseminated capacity in the

body, filamentous forms are those with greater tissue penetrating capacity, capable of causing serious damage to the host [42-44].
Therefore, it is desirable, to search compounds capable of acting in this morphological transition, preventing the generation of hyphal forms. At present, scientific research offers different molecules with these characteristics, such as phenazines and homoserinic lactones derived from *Pseudomonas aeruginosa*, capric acid secreted by *Saccharomyces boulardii*, or farnesol produced by *Candida albicans* [39].

Farnesol
This molecule has been the subject of extensive research since it was discovered that this compound was able to inhibit both the filamentation and biofilm formation in *Candida albicans*, the virulence factors [9, 45].
In an *in vitro* study that appeared in The Open Microbiology Journal in 2011, Decanis and colleagues have shown that this alcohol was able to inhibit the generation of hyphal forms of *Candida albicans* [46]. Through the results of this study the following evidence emerged: the least formation of germ tubes in the cells of *Candida albicans* treated with farnesol in respect to the control (untreated cells), an effect which manifests itself for any concentration of farnesol, the rapidity of action and the maintaining the inhibitory effect of the compound against hyphal forms of *Candida albicans* even after 24 hours [46].

Discoveries in the making
Sze Wah Wong and colleagues, in a recent study, which appeared in Plos One in 2014, have identified a molecule from a low molecular weight, with an activity both *in vitro* and *in vivo* against *Candida* spp. [47].
This molecule, called SM21 by the working group, interferes with both virulence factors, expounding, in fact, its activity against the filamentation process and inhibiting the formation of biofilms. The molecule, also, seems to be endowed with peculiar properties, proving, among other things also active against *Candida* spp. resistant to antifungal therapy, and is characterized by a low toxicity profile in respect of human cells [47].
The way leading to the use of drugs in clinical practice is long. Whether clinical trial results should confirm the favorable outcome of the molecule so far tested, this compound, after a market authorization and others step, may be widely used in medical practice [47].
The research for compounds able to counteract the filamentation process in *Candida albicans* is not limited to just the above described work. In another study, which appeared in Plos One in 2011, Midkiff and colleagues found other molecules capable of acting in this important morphologic transition [48]. Unlike the contribution from Sze Wah Wong and colleagues that have also the compound *in vivo* tested, the majority of these works has been *in vitro* testing only, not allowing a first assessment of the efficacy and toxicity of the compound in animal models, similar to primates [39]. Research is progressing on this front: counteracting the virulence factors of *Candida* spp. seems the way to follow to avoid the negative consequences that would arise on the host [39].

Additional therapeutic targets
The fungal cell wall represents a potential target for antifungal drugs. In fact, it is unique in its kind and the molecules that interfere with this structure directly kill the fungus, for cell lysis [50]. Since the fungal protein kinases regulate the biosynthesis of the cell wall, the scientific research is shifting towards the discovery of compounds capable of inhibiting this signaling pathway [49]. In a contribution published in ACS Chem Biol. in 2011, Baxter and colleagues have identified a few compounds with inhibitory activity against Phosphoinositide-dependent-1 kinase, as well as active both against planktonic forms and towards fungal biofilms [50].
Two inhibitors of these protein kinase, called OSU-03012 and UCN-01, have already passed the *in vitro* and in animals tests and are now in the clinical trial phase [50].
It is believed that these compounds, from the innovative mechanism of action, could represent a further step forward in infectious disease research, in a historical period characterized by the increasing resistance of fungal species to drug treatment [50].

CONCLUSIONS

Nosocomial infections and particularly those biofilm-associated, even more difficult to treat since

they are almost impenetrable to drug treatment, generate serious consequences for health. The dramatic nature of the phenomenon is given by the fact that the antifungal drugs used in an associated biofilm infection are limited, especially when compared to a richer arsenal of compounds capable of acting towards a bacterial biofilm. Moreover, the situation is further aggravated by the fact that the fungal species, enclosed within the microbial consortium, are proving increasingly resistant to drug treatment.

The search for innovative molecules, able to counteract this phenomenon, is vivid; it is desirable to follow this perspective which guides the identification of effective compounds, capable of overcoming the growing phenomenon of drug resistance, so that they can find a use in clinical practice in the near future.

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