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In vitro interactions between Anidulafungin and nonsteroidal anti-inflammatory drugs on biofilms of Candida spp.

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Abbreviations: ANF, anidulafungin; ASA, aspirin; COX, cyclo-oxygenase; DFCN, diclofenac; DMSO, dimethyl sulfoxide; DO, optical density; ECs, Echinocandins; FIC, Fractional inhibitory concentration; FICI, Fractional inhibitory concentration indice; IBF, ibuprofen; MIC, minimum inhibitory concentration; NSAID, non-steroidal anti-inflammatory drug; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide

Abstract

Candida spp. are responsible for many biomaterial-related infections; they give rise to infective pathologies typically associated with biofilm formation. We recently reported that the echinocandin anidulafungin (ANF) showed a strong *in vitro* activity against both planktonic and biofilms cells. Herein, we report the antifungal activities of ANF alone and in association with some non-steroidal anti-inflammatory drugs (NSAIDs) against nine *Candida* strain biofilms: four *C. albicans*, two *C. glabrata and* three *C. guilliermondii.* The activity of ANF was assessed using an *in vitro* microbiological model relevant for clinical practice. ANF proved oneself to be active against biofilms cells, and a clear-cut synergism was found against *Candida* species biofilms when ANF was used in combination with three NSAIDs: aspirin, diclofenac, ibuprofen. The positive synergism against *Candida* spp. of ANF in association with aspirin or the other NSAIDs proved to be a very effective antifungal treatment (FICI < 0.5). These results may provide the starting point for new combination therapies of ANF with NSAIDs against *Candida* biofilm pathologies.

Keywords: biofilm, non-steroidal anti-inflammatory drugs (NSAIDs), anidulafungin, XTT, synergism, *Candida* species.

1. Introduction

Some pathogenic *Candida* species cause serious superficial and systemic infections widely recognized in modern clinical practice.[1–3] It is well documented that most *Candida* infections involve biofilm formation[4,5] on implanted devices (indwelling catheters) and tissue surfaces, which facilitates adhesion of the yeast to the host surface or to an associated prosthesis, such as a denture or intravascular catheter.[6] Opportunistic yeast infections such as candidiasis or *Candida* biofilm particularly occur in immunocompromised patients;[7] thus, the treatment of these infections represents a serious problem for contemporary medicine.[8] Nowadays, only a few antifungal agents are available, and their effectiveness is not always optimal as many of them can cause toxicity and resistance.[9–13] Several research works evidenced *Candida* species as planktonic or sessile cells (biofilms) to be strongly resistant to a wide spectrum of conventional antifungal agents, for example azoles. In particular, *Candida* biofilms are reported to be resistant to the new triazoles, voriconazole and ravuconazole, which have an extended spectrum of activity against many azole-resistant organisms as well as fungicidal, rather than fungistatic, activity.[14,15] The failure of antifungal therapy leads to chronic infections that may be cured only by surgery and/or removal of implants. Actually, therapy based on combination of drugs could represent a promising perspective to definitively solve this important aspect.[16–18] Echinocandins (ECs) are antifungal agents that inhibit the synthesis of 1,3-β-D-glucan, a key component of the cell walls of several pathogenic fungi. They act against *Candida* spp. biofilm-associated infections, which are frequently refractory to conventional therapy.[19–21] Several studies have been carried out, mainly on the use of ECs, acting on *C. albicans* and *C.* non-*albicans* biofilms.[22,23] A comprehensive report, which compares the *in vitro* activities of three ECs (anidulafungin, caspofungin and micafungin) against biofilms formed by different non-*Candida* species showed that anidulafungin (ANF), a semi-synthetic lipopeptide, had the better efficacy of the three antifungal drugs studied.[24–26] This compound has demonstrated antifungal activity against many amphotericin B-resistant *Candida* spp., too. Recently, we reported that ANF showed a strong *in vitro* activity against both planktonic and biofilms cells and we confirmed that high ANF concentrations

might establish paradoxical growth effect in *C. albicans* and *C. tropicalis* biofilms.[27] Among the strategies to eradicate fungal biofilms of different *Candida* spp., the use of ECs in combination with other antifungal agents has been proposed.[28] We previously reported the in vitro synergy tests of ANF with other antifungal agents.[29] It has been demonstrated that mammalian cells and pathogenic fungi as *Criptococcus* or *Candida* have the capacity to produce prostaglandins directly or by synthesis from exogenous arachidonic acid.[30–34] Prostaglandin are small lipid molecules with some different activities for the mammalian metabolism such as the modulation of immune response. Thus, drugs able to inhibit prostaglandin synthesis, as the well known non-steroidal anti-inflammatory drugs (NSAIDs), may play an important biochemical role that could affect prostaglandins fungal metabolism. Alem and Douglas clarified the inhibitory effect of some NSAIDs (aspirin, diclofenac, and etodolac) on *Candida* biofilms: diclofenac sodium had the greatest inhibitory impact on the growth of *Candida* biofilms.[35] The significant activity of diclofenac against fungal biofilms was successively confirmed.[36–38] Diclofenac seems also to potentiate the *in vivo* activity of caspofungin against *C. albicans* biofilm.[39] Moreover, a combination of fluconazole and NSAIDs results in synergistic activity against *C. albicans*.[40,41] On the other hand, in our knowledge, no studies are available about the combination of ANF with currently used NSAIDs against *Candida* spp. biofilms. Our research work was focused on the synergistic *in vitro* effect of ANF combinations with NSAIDs (aspirin, diclofenac, ibuprofen) against nine *Candida* ATCC strains as biofilms. Since colonization often begins with the appearance of a biofilm, herein we describe a quantitative method for the determination of the inhibitory percentage toward the control and Fractional Inhibitory Index for the synergistic interaction, in order to evaluate this effect. To highlight the effectiveness of associations of NSAIDs with ANF *in vitro* against different *Candida* strain biofilms, chequerboard method has been used.[42,43] These combinations should improve the management of *Candida* biofilm-associated infections disturb the biofilms and avoid the emergence of resistance.[44]

2. Results and discussion

Three NSAIDs (aspirin, diclofenac, ibuprofen) and the antifungal echinocandin ANF were evaluated on a large panel of yeasts. The most interesting results were those found against nine *Candida* strain biofilms and are reported in Table 1 and 2. The effect of the combination of ANF with each of the three NSAIDs was also evaluated. MIC value of ANF against all strains was found to be 2 µg/ml in our described experimental conditions, while the strains were susceptible to NSAIDs at concentrations ranging between 0.2 and 100 mM. The inhibition of the biofilms growth by aspirin was more evident at concentrations ranged between 0.2 mM and 1 mM; after 24 h of incubation with 1 mM of aspirin, for considered strains (*C. albicans* ATCC 90028, *C. albicans* ATCC 24433, *C. guilliermondii* ATCC 6260, *C. guilliermondii* ATCC a410), biofilm activity was considerably reduced (44.7%, 48.7%, 54.1%, 43.2%, respectively, Tab. 2). Ibuprofen showed a lower inhibition activity than aspirin against *C. albicans* strain biofilms (ranging between 16.3 and 25.6% at 1 mM), while its activity against *C. glabrata* and *C. guilliermondii* was more pronounced (ranging between 53.2 and 64.4% at 0.2 mM). The other NSAID diclofenac inhibited the tested biofilms to a lesser level at 100 mM. ANF inhibited biofilms but did not cause a significant decrease in the growth of the biofilm respect to the action of the single NSAIDs tested. Furthermore, the different concentrations inhibited biofilm viability for all the tested strains, and DO (Optical density) of the control strains did not increase for 48 h of incubation. The chequerboard assay method for combinations activity of ANF and NSAIDs showed for *C. albicans* a decrease of SMIC of the four strains towards ANF up to 1/20 MIC of ANF. Even SMICs of NSAIDs were significantly reduced when they were used in combination with ANF against the species of *Candida* used: in fact, all the three NSAIDs showed a progressive reduction up to 1/20 of SMICs from the previous test (when NSAIDs were used alone); precisely between 1/10 and 1/20 for aspirin, 1/5 and 1/20 for diclofenac, 1/5 and 1/10 for ibuprofen. The NSAIDs FIC indices ranged between 0.10 and 0.15, up 0.10 to 0.45, up 0.25 to 0.30, for aspirin, ibuprofen, diclofenac, respectively. These results have a high significance for a strong synergistic activity against almost all biofilm strains. It is worthy of note that the better results of combinations

of ANF and NSAIDs were obtained with aspirin. The combinations of NSAIDs and ANF studied did not show a strong effect against *C. guilliermondii* ATCC 6260. These strains showed a percentage reduction of biofilm growth lower than the inhibition percentages of the single drugs. Our results confirmed the different sensitivity of *Candida* spp. to drugs. *C. albicans* and *C. glabrata* were found to be very sensitive to ANF and NSAIDs while a weak synergistic effect was found for the combinations of ANF with NSAIDs against *C. guilliermondii*. Actually, Table 1 and 2 resume the results of this checkerboard analysis. The results given above related to our isolates and ATCC strains biofilm, as reported in this paper, show that *in vitro* therapeutic combinations of ANF with NSAIDs have a strong effect on *C. albicans* and *C. glabrata* biofilms. From our point of view, the synergistic effect observed in our experiments is conceivably due to the probable lack of β-1,3-D-glucan induced by ANF which increases the amount of NSAID able to reach the membrane; this effect should give NSAID access to the cell membrane and as consequence the possibility to block the prostaglandin production. Our experimental data suggest an effective alternative solution to managing of biofilm-associated infections. Even though these results may be considered a preliminary report, the synergism between ANF and NSAIDs, more evident than the one reported in other interaction studies with other antifungal agents as fluconazole, deserves to be highlighted. Furthermore, among the NSAIDs considered in our research, aspirin significantly reduced the viability of cells biofilm, confirming what was previously reported by Alem and Douglas who underlined the possibility that aspirin has a greater effect on viability than biofilm formation.[35]

3. Conclusion

The goal of this study was the *in vitro* evaluation of synergistic effects of NSAIDs with ANF against *Candida* biofilm at different concentrations, using the XTT microtiter method and FIC index. It was demonstrated that the well-known prostaglandin synthesis inhibitors ibuprofen, diclofenac and particularly aspirin, in combination with ANF, strongly decrease biofilm production by several

Candida spp. ANF and NSAID biofilm MICs were significantly lowered when their associations were tested. Among the NSAIDs considered in our research, aspirin significantly reduced the viability of cells biofilm. Since in our knowledge no studies have investigated the synergy between NSAID and ANF against clinical isolates causing invasive infections, so far, our results may represent an interesting starting point for an alternative route to new synergistic antifungal therapies against biofilm infections, overcoming the high cost of new drugs and the potential risk of antagonistic interactions.

4. Materials and Methods

Organisms

A total of 9 yeast strains were used in this study: four *Candida albicans,* two *Candida glabrata and* three *Candida guilliermondii: C. albicans* ATCC 10231, *C. albicans* ATCC 90028, *C. albicans* ATCC 24433, *C. albicans* 17a18, *C. glabrata* ATCC 15126, *C. glabrata* 18a10, *C. guilliermondii* ATCC 6260, *C. guilliermondii* a83, *C. guilliermondii* a410. The isolates were from the blood of patients with candidemia admitted to the intensive care unit of Department of Biomedical Science and Human Oncology of Bari – Italy. They were identified by sugar assimilation profiles, using the biochemical tests performed with the commercial system API ID32C (BioMerieux, Marcy l'Etoile-France). Stocks were maintained at –80 °C in yeast peptone dextrose broth with 10% to 25% glycerol (Oxoid, Italy) solution. All strains were stored at –20 °C in glycerol stocks, and were subcultured on antimicrobial agent-free Sabouraud Dextrose Agar plates (BioMerieux, Marcy l'Etoile-France) to ensure viability and purity before the start of the study.

Medium and culture conditions

Each frozen stock culture was inoculated onto Sabouraud Dextrose Broth (Difco, Milan, Italy) and incubated at 37 °C for 24 h, in an orbital shaker at 60 rpm. Cells were picked up and added to a tube

containing RPMI 1640 broth medium with L-glutamine and without bicarbonate buffered to pH 7 with MOPS, 3-*(N*-morpholino)propanesulfonic acid (165 M, Sigma, Italy). A standardized suspension of $1 \cdot 10^6$ CFU/ml was obtained and immediately used.

Prostaglandin inhibitors

The three NSAIDs used were purchased from Sigma–Aldrich, Milan, Italy. Stock solutions (100 mM) of diclofenac, ibuprofen were prepared in dimethyl sulfoxide. The stock solution of aspirin was prepared in ethanol. In biofilm experiments COX inhibitors were used at a final concentration ranging from 0.1 μM to 100 mM.

Antifungal agent and material

Pfizer inc. (Rome, Italy) supplied ANF, and 13-mm diameter PVC flat disks with a thickness of 2 mm were obtained from Mediatipo (Bari, Italy). The disks were washed in dilute laboratory soap, rinsed three times in distilled water and ethanol 30%, air dried, and sterilized in an autoclave before use.

Biofilm formation and testing

Autoclaved disks, containing 2 ml of $1 \cdot 10^6$ cfu ml^{-1} *Candida* cells suspension, were placed one disk per well in 24 wells tissue culture plates (Corning Inc, Corning, Milan). The plates containing disks were incubated at 37 °C for 1.5 h with shaking at 100 rpm on a rocker table enabling the *Candida* cells attachment (adhesion phase). Afterwards the disks were gently washed three times with sterile PBS, transferred to a new 24 wells plate containing 2 ml of RPMI 1640 and incubated for 48 h at 37 °C, allowing the biofilm formation (maturation phase). Control disks were handled in an identical way except that no fungal cells were added.

Quantification of biofilms growth

From an experimental point of view, SMICs endpoints were determined colorimetrically by using XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide, a reduction assay indicator in 24-well polystyrene microtiter plate. We used *in vitro* XTT to determine ANF synergism activity against ATCC and isolates of fungal strain biofilms [45]. The procedure simplifies biofilm formation and quantification, making it more reliable and easier to compare between different laboratories and this is a necessary step toward the standardization of antifungal susceptibility testing of biofilms. As previously published by others we assumed that data based on XTT metabolic activity was sufficient to indirectly quantify biofilms. Quantification of biofilm growth was performed colorimetrically by a solution of XTT with menadione as electron coupling agent. Each day XTT Menadione solution was prepared in 2 ml of sterile PBS by adding 180 µl of XTT (1 mg/ml in DMSO; Sigma chemicals, Milan, Italy) to 30 µl of Menadione solution (0.4 mM in DMSO; Sigma chemicals, Milan, Italy). Disks containing *Candida* sp. biofilms were washed and transferred to a new 24 wells plate containing 2 ml of sterile PBS and 180 µl of XTT-Menadione solution per well (as described above). Plates were incubated at 37°C for 2h, and the medium was removed and centrifuged for 10 min at 3.000 × g to pellet any suspended cells or debris. The amount of XTT-Formazan in the supernatant was measured at 490 nm by using a spectrophotomer (Genesys 20, Thermo Electron Corporation). For susceptibility testing (SMIC), biofilms were formed in RPMI 1640 medium as described above. After 48 h preformed biofilm containing disks were washed three times with sterile PBS prior to test with ANF and NSAIDs combinations diluted in RPMI 1640 medium. After exposure to different concentrations of the antifungal agents ANF and NSAID, for 24 h at 37 °C on a rocker table, biofilm activity was measured by XTT reduction assay as described above. In vitro synergism between NSAIDs and ANF was made using a two dimensional checkerboard dilution technique in sterile 24 wells flat bottom plates, containing the solution of the two components to be tested. The concentrations of antifungal combinations causing reduction a 50% of metabolic activity compared with the metabolic activity of the drug-free control was then determined (SMICs). Antimicrobial susceptibility tests of biofilms

were performed in triplicate in different days. In our experimental protocols, the combinations of the substances were analyzed by calculating FIC index (FICI). Generally, FICI value was interpreted as: i) a synergistic effect when it is ≤ 0.5; ii) an additive or indifferent effect when it is > 0.5 and <1; iii) an antagonistic effect when it is > 1.[29]

Conflict of interest: the authors declare no conflict of interest.

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Table 1. Non steroidal anti-inflammatory drugs (NSAIDs) and Anidulafungin - Fractional inhibitory concentration (FIC) and FIC indices (FICI)

	Aspirin				Diclofenac				Ibuprofen			
	MICo	**MICc**	**FIC**	**FICI**	**MICo**	**MICc**	**FIC**	**FICI**	**MICo**	**MICc**	**FIC**	**FICI**
***Candida albicans* ATCC 10231**												
NSAID (mM)	0.20	0.01	0.05	0.10	100	5.0	0.05	0.10	1.0	0.05	0.05	0.25
Anidulafungin (µg/ml)	2.0	0.10	0.05		2.0	0.10	0.05		2.0	0.4	0.20	
***Candida albicans* ATCC 90028**												
NSAID (mM)	1.0	0.01	0.05	0.10	100	5.0	0.05	0.10	1.0	1.0	0.10	0.30
Anidulafungin (µg/ml)	2.0	0.10	0.05		2.0	0.10	0.05		2.0	0.40	0.20	
***Candida albicans* ATCC 24433**												
NSAID (mM)	1.0	0.10	0.10	0.15	100	5.0	0.05	0.10	1.0	1.0	0.10	0.30
Anidulafungin (µg/ml)	2.0	0.10	0.05		2.0	0.10	0.05		2.0	0.40	0.20	
***Candida albicans* 17a18**												
NSAID (mM)	0.20	0.01	0.05	0.10	100	5.0	0.05	0.10	1.0	0.05	0.05	0.25
Anidulafungin (µg/ml)	2.0	0.10	0.05		2.0	0.10	0.05		2.0	0.4	0.20	
***Candida glabrata* ATCC 15126**												
NSAID (mM)	0.20	0.01	0.05	0.10	100	20	0.20	0.30	0.20	0.04	0.20	0.25
Anidulafungin (µg/ml)	2.0	0.10	0.05		2.0	0.20	0.10		2.0	0.10	0.05	
***Candida glabrata* 18a10**												
NSAID (mM)	0.20	0.01	0.05	0.10	100	20	0.20	0.30	0.20	0.04	0.20	0.25
Anidulafungin (µg/ml)	2.0	0.10	0.05		2.0	0.20	0.10		2.0	0.10	0.05	
***Candida guilliermondii* ATCC 6260**												
NSAID (mM)	1.0	0.10	0.10	0.15	100	40	0.40	0.45	0.20	0.02	0.10	0.30
Anidulafungin (µg/ml)	2.0	0.10	0.05		2.0	0.10	0.05		2.0	0.40	0.20	
***Candida guilliermondii* a83**												
NSAID (mM)	0.20	0.01	0.05	0.10	100	20	0.20	0.30	0.20	0.04	0.20	0.25
Anidulafungin (µg/ml)	2.0	0.10	0.05		2.0	0.20	0.10		2.0	0.10	0.05	
***Candida guilliermondii* a410**												
NSAID (mM)	1.0	0.10	0.10	0.15	100	40	0.40	0.45	0.20	0.02	0.10	0.30
Anidulafungin (µg/ml)	2.0	0.10	0.05		2.0	0.10	0.05		2.0	0.40	0.20	

MICo =MIC of an individual sample; MICc= MIC of an individual sample at the most effective combination; FIC= Fractional Inhibitory Concentration (see text); FICI = FIC of NSAID + FIC of Anidulafungin

Table 2. Synergistic effects of COX inhibitors (NSAIDs) in combination with Anidulafungin on biofilm formation of *Candida* spp.[a]

XTT reduction (%) a by biofilms after 48 h							
Strain	ANF	ASA	ASA-ANF	DFCN	DFCN-ANF	IBF	IBF-ANF
Candida albicans ATCC 10231	33,98	49,21	82,81	53,57	66,15	16,26	59,51
Candida albicans ATCC 90028	16,09	44,72	57,84	51,15	71,04	25,62	61,43
Candida albicans ATCC 24433	25,06	48,75	56,14	50,25	65,24	22,45	60,14
Candida albicans 17a18	29,31	46,55	51,74	55,33	64,25	24,51	64,22
Candida glabrata ATCC 15126	27,41	29,02	49,78	54,44	65,96	57,23	57,46
Candida glabrata 18a10	37,42	32,32	48,58	44,54	58,66	53,44	62,33
Candida guilliermondii ATCC 6260	51,21	54,12	50,37	60,98	57,69	64,43	53,32
Candida guilliermondii a83	47,83	46,22	45,32	57,87	55,64	56,78	52,11
Candida guilliermondii a410	45,62	43,25	41,54	58,69	54,28	53,24	51,31

[a]ANF = Anidulafungin; ASA = Aspirin; DFCN = Diclofenac, IBF = Ibuprofen.

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