Peptides 71 (2015) 211–221

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Antifungal activity of cathelicidin peptides against planktonic and biofilm cultures of *Candida* species isolated from vaginal infections

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ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 23 July 2015
Accepted 24 July 2015
Available online 31 July 2015

Keywords:
Cathelicidin
Alfa-helical antimicrobial peptide
Candida albicans
Biofilm
Candida isolates
Vulvovaginal candidiasis

ABSTRACT

Vulvovaginal candidiasis (VVC) is a frequent gynecological condition caused by *Candida albicans* and a few non-*albicans Candida* spp. It has a significant impact on the quality of life of the affected women also due to a considerable incidence of recurrent infections that are difficult to treat. The formation of fungal biofilm may contribute to the problematic management of recurrent VVC due to the intrinsic resistance of sessile cells to the currently available antifungals. Thus, alternative approaches for the prevention and control of biofilm-related infections are urgently needed. In this regard, the cationic antimicrobial peptides (AMPs) of the innate immunity are potential candidates for the development of novel antimicrobials as many of them display activity against biofilm formed by various microbial species. In the present study, we investigated the in vitro antifungal activities of the cathelicidin peptides LL-37 and BMAP-28 against pathogenic *Candida* spp. also including *C. albicans*, isolated from vaginal infections, and against *C. albicans* SC5314 as a reference strain. The antimicrobial activity was evaluated against planktonic and biofilm-grown *Candida* cells by using microdilution susceptibility and XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide] reduction assays and, in the case of established biofilms, also by CFU enumeration and fluorescence microscopy. BMAP-28 was effective against planktonically grown yeasts in standard medium (MIC range, 2–32 μM), and against isolates of *C. albicans* and *Candida krusei* in synthetic vaginal simulated fluid (MIC range 8–32 μM, depending on the pH of the medium). Established 48-h old biofilms formed by *C. albicans* SC5314 and *C. albicans* and *C. krusei* isolates were 70–90% inhibited within 24 h incubation with 16 μM BMAP-28. As shown by propidium dye uptake and CFU enumeration, BMAP-28 at 32 μM killed sessile *C. albicans* SC5314 by membrane permeabilization with a faster killing kinetics compared to 32 μM miconazole (80–85% reduced biofilm viability in 90 min vs 48 h). In addition, BMAP-28 at 16 μM prevented *Candida* biofilm formation on polystyrene and medical grade silicone surfaces by causing a >90% reduction in the viability of planktonic cells in 30 min. LL-37 was overall less effective than BMAP-28 against planktonic *Candida* spp. (MIC range 4–≥64 μM), and was ineffective against established *Candida* biofilms. However, LL-37 at 64 μM prevented *Candida* biofilm development by inhibiting cell adhesion to polystyrene and silicone surfaces. Finally, *Candida* adhesion was strongly inhibited when silicone was pre-coated with a layer of BMAP-28 or LL-37, encouraging further studies for the development of peptide-based antimicrobial coatings.

Abbreviations: AMB, amphotericin B; CFU, colony forming unit; CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; FLC, fluconazole; ITC, itraconazole; MCZ, miconazole; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration; PBS, phosphate buffered saline; SE, silicone elastomer; VSF, vaginal simulated fluid; VVC, vulvovaginal candidiasis; XTT, [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide].

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http://dx.doi.org/10.1016/j.peptides.2015.07.023
0196-9781/

1. Introduction

Mucosal infections caused by *Candida* spp., particularly *Candida albicans*, are very common, and vulvovaginal candidiasis (VVC) is the second most frequent gynecological condition after bacterial vaginosis being diagnosed in a considerable part of women with vaginitis [1]. Results from a recent internet panel survey involving 6000 women in the US and Europe indicate an overall rate of VVC ranging from 29% to 49%, with four or more recurrences over a 12-month period in more than one fifth of the cases [23]. VVC results from overgrowth of various *Candida* species, usually present in vagina as commensals, in otherwise healthy women [68]. The infection is mainly caused by *C. albicans* (85–95% of cases) and a few non-*albicans Candida* spp [68]. The prevalence of the latter species varies widely in different parts of the world and among them, *Candida glabrata* is the most common [1]. Several studies report higher percentages of non-*C. albicans* species from women with recurrent VVC, HIV-infected or post-menopausal women, and women with uncontrolled diabetes [1]. Particularly, VVC is not a life-threatening condition, yet it has a significant impact on the quality of life of the affected women [1]. The standard treatment of uncomplicated VVC is principally based on oral or topically applied azoles, that are effective in more than 90% of *C. albicans* infections [1]. On the contrary, treatment of complicated VVC such as recurrent VVC and that caused by non-*C. albicans* species is still challenging mainly due to reduced azole susceptibility of non-*C. albicans* species and to persistence of *C. albicans* on the external vulva in patients with recurrent VVC after the cessation of treatment [1,6].

C. albicans is a polymorphic yeast which associates with natural surfaces, e.g., epithelial or endothelial cells, as well as with abiotic surfaces, such as central venous and urinary catheters, forming biofilms [40]. The biofilm forming ability is considered as a virulence factor since the sessile lifestyle provides protection against the host immune system and results in fungal resistance to antimycotics [1,46,60]. This is particularly worrisome in hospital settings, considering that *Candida* spp. are able to form biofilms on virtually any implanted medical device in the human host and that *C. albicans* is the most common fungal organism associated with biofilm-related infections [12,72].

Candida biofilms may contribute to the pathogenesis of superficial as well as systemic *Candida* infections. In this respect, *C. albicans* strains isolated both from blood and mucosa showed similar in vitro biofilm formation capacity, without significant difference between invasive and non invasive isolates, despite high intra-species variability [31,71]. Concerning gynecological infections, *C. albicans* and other *Candida* spp., isolated from VVC patients, displayed in vitro the ability to adhere both to the plastic of microtiter plates and to the surface of intrauterine contraceptive devices, producing biofilm to different extents [11,56,57]. In an experimental mouse model of *Candida* vaginitis, Harriott et al. reported the presence of sessile *C. albicans* attached to the vaginal mucosa and encased in a typical biofilm architecture with abundant extracellular matrix, which became evident after 24–48 h of infection [30]. They further demonstrated in a recent in vivo study that key transcriptional regulators controlling the yeast-to-hypha switch are implicated in the immunopathogenesis of vaginal candidiasis and in the transition from asymptomatic colonization to symptomatic infection [58]. In addition, Auler et al. isolated *C. albicans* from intrauterine devices that have been removed from patients with recurrent VVC, thus indicating the presence of biofilm on the contraceptive device as a risk factor for recurrence [4]. Recurrent VVC is considered a multifactorial disease and the formation of biofilm may contribute to the problematic management of this condition due to the intrinsic resistance of sessile cells to commonly used antifungal drugs [60].

Alternative approaches for the prevention and control of biofilm-related infections are urgently needed. In the last two decades, the cationic antimicrobial peptides (AMPs) of the innate immunity have received increasing attention as potential candidates for the development of novel antimicrobials [22,29,87]. Considerable interest in these molecules is based on their potent broad-spectrum antimicrobial activity, low propensity to induce resistance, and ability to modulate the host immune system [64,84,85]. These natural peptides are part of the first line defense in a wide range of organisms including mammals [87]. Apart from being produced by professional phagocytes, they are found on the skin and on the epithelia lining the respiratory, the gastrointestinal and genitourinary tract where they provide protection against infection [50,64]. One of the best characterized AMPs in humans is the cathelicidin LL-37 [78,86], a linear cationic alfa-helical peptide that has been identified also in the squamous epithelium of the cervix and vagina [26] and in cervicovaginal secretions. Increased levels of LL-37, among a few other AMPs, were found in vaginal fluids of women affected by multiple ongoing vaginal infections including candidiasis [43]. Collectively, these data would suggest a role for this peptide in antimicrobial defence of the vaginal epithelium. Recently, Lan and co-workers have shown that LL-37 inhibits the adhesion of *C. albicans* to plastic surfaces and epithelial cells by interfering with yeast carbohydrate and protein cell-wall components [10,77]. As microbial adhesion represents a crucial step in biofilm formation and the ability of LL-37 to inhibit the development of bacterial biofilm is well characterized [36,54], we asked ourselves whether LL-37 was also active against biofilm formed by *C. albicans*. We were further interested in comparison between LL-37 and the bovine ortholog BMAP-28, a cathelicidin peptide [85] highly active against a broad spectrum of pathogens including yeasts and filamentous fungi [7,8,74,76]. BMAP-28 was recently shown to be active against bacterial biofilm formed by cystic fibrosis isolates [59], but its activity against fungal biofilm has not been studied so far. Although both are linear helical peptides, the differences in their structuring and aggregational properties [48] could affect their antifungal specificities.

In the present study, we investigated the antifungal activity of LL-37 and BMAP-28 against pathogenic *C. albicans* and non-*albicans Candida* spp. with reduced azole-susceptibility, isolated from vaginal infections. XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] reduction assays have been used to evaluate the ability of these peptides to inactivate yeast cells in the planktonic state, to prevent yeast adhesion to microtiter plates and medical grade silicone surfaces, and to eradicate established biofilms. Biofilm killing was further confirmed by colony counts. The effects of the AMPs on fungal membrane permeability in target biofilms were addressed by measuring propidium dye uptake by fluorescence microscopy. The anti-biofilm activity of the AMPs under study was evaluated in comparison with that of the polyene amphotericin B (AMB) and the imidazole miconazole (MCZ).

2. Materials and methods

2.1. Antimicrobial peptides and drugs

Peptides (BMAP-28, GGLRSLGRKILRAWKKYGPIIVPIIRI-NH_2; LL-37, LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) were chemically synthesized according to standard methods [75] and confirmed by mass spectrometry using a Q-STAR hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with an electrospray ion source. Peptide concentrations were determined in aqueous solution by measuring the absorbance at 257 nm (Phe residues) and 280 nm (Tyr and Trp residues) for LL-37 and BMAP-28, respectively [75].