Peptides 32 (2011) 99–103

Contents lists available at ScienceDirect

Peptides

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

In vitro activity of the lipopeptide PAL-Lys-Lys-NH2, alone and in combination with antifungal agents, against clinical isolates of *Candida* spp.

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ARTICLE INFO

Article history:
Received 15 June 2010
Received in revised form 20 October 2010
Accepted 20 October 2010
Available online 3 November 2010

Keywords:
Candida spp.
PAL-Lys-Lys-NH2
Amphotericin B
Caspofungin
Fluconazole
Synergy
In vitro

ABSTRACT

Candida albicans is known to be the organism most often associated with serious fungal infection, but other *Candida* spp. are emerging as clinical pathogens associated with opportunistic infections. Among antimycotic treatments, increasing attention is currently given to anti-infective drugs based upon naturally occurring peptides, such as the short lipopeptide palmitoyl PAL-Lys-Lys-NH2 (PAL). The aim of this study is to evaluate the activity of this peptide compared to the traditional antifungal agents Fluconazole (FLU), amphotericin B (AMB) and caspofungin (CAS) on *Candida* spp. 24 clinical isolates of *Candida* spp. were tested against PAL, FLU, AMB and CAS using *in vitro* susceptibility tests, time killing and checkerboard assay. All of the drugs studied showed good activity against clinical isolates of candida; in particular CAS and AMB which have MICs value lower than PAL and FLU. Moreover we observed synergistic interactions for PAL/FLU (81.25%), PAL/AMB (75%) and particularly for PAL/CAS (87.5). We think that our results are interesting since synergy between PAL and CAS might be useful in clinic trails to treat invasive fungal infections.

1. Introduction

Over the past few decades, candida infections have become dramatically significant and the need for early implementation of antifungal agents increases in parallel with the expanding number of *Candida* spp. exhibiting resistance to traditional triazole antifungals like fluconazole, and cross resistance with newer triazoles. *Candida albicans* is known to be the organism most often associated with serious fungal infection, but other *Candida* spp. are emerging as clinical pathogens associated with opportunistic infections [29,35]. The frequency of *Candida glabrata* as the cause of severe fungal infections has increased worldwide. On the other hand *C. parapsilosis* is the *Candida* species which is the second most frequently found in children as well as a frequent cause of endocarditis among parenteral drug abusers [6,7,29]. Candidiasis has been associated with increased overall and attributable mortality and morbidity, longer duration of intensive-care units stay, and increased costs [14,37]. The choice of the proper treatment is determined by the site and extent of the infection, the etiological agent, as well as by the efficacy, safety, profile and kinetics of available drugs [3,33]. Amphotericin B deoxycholate (AMB), a polyene, which bind to mature membrane sterols has been considered the "gold standard" for the treatment of invasive fungal infections for more than two decades. Although the high toxicity of this drug has somewhat limited its use [27]. Oral azoles, which inhibit sterol formation, are the antifungal agents currently used to treat severe infections. In recent years, increasing usage of fluconazole (FLU), has led to changes in the prevalence of *Candida* spp. and the selection of azole resistant strains [4,13]. Recently echinocandines, like caspofungin (CAS) are used against mucocutaneous and invasive candidiasis. The drug exhibits concentration-dependent fungicidal or fungistatic activity against several *Candida* species [11,12]. In the recent years several new drugs have emerged as possible therapeutic alternatives, and among the compounds that are currently under investigation for their therapeutic potentials are antimicrobial peptides (AMPs) of the innate immune system and their synthetic derivatives [18,19].

In particular lipopeptides are produced only in bacteria and fungi during cultivation on various carbon sources [10]. They are composed of a specific lipophilic moiety attached to an anionic peptide (six to seven amino acids [23]. They have an efficient and broad spectrum of activity toward both bacteria and fungi and act on the

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doi:10.1016/j.peptides.2010.10.022

membranes of the microorganisms via a lytic mechanism by which it should be difficult to confer resistance [19,20,32].

Increasing attention is currently given to a short lipoptide palmitoyl PAL-Lys-Lys-NH2 (PAL) [1,5]. The aim of this study is to evaluate the activity of this peptide compared to the traditional antifungal agents FLU, AMB and CAS against 24 clinical isolates of *Candida* spp. using *in vitro* susceptibility tests, time killing and checkerboard assay.

2. Materials and methods

2.1. Yeast isolates

A total of 24 *Candida* spp. isolates were investigated, they included 4 strains of *Candida albicans*, 4 of *Candida parapsilosis*, 4 of *Candida glabrata*, 4 of *Candida krusei*, 4 of *Candida tropicalis* and 4 of *Candida guilliermondii*. *Candida albicans* ATCC 90029, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750 and *Candida krusei* ATCC 6258 were included as reference strains. The isolates were recovered from blood, the gastrointestinal tract, the respiratory tract, the urinary tract, and from lesions of nails in patients attending the Clinical of Infectious Disease and the Clinic of Dermatology, University of Ancona, Italy. Each strain represented a unique isolate from a given patient. Yeasts isolates were identified at the species level by conventional morphological and biochemical methods and stored at −70 °C in 10% glycerol. Prior to testing, isolates were subcultured twice on Sabouraud dextrose agar (SDA) plates.

2.2. Antifungal agents

PAL was synthesized manually by the solid-phase methodology using the Fmoc/tBu strategy. The peptide amide was prepared on Polystyrene AM-RAM resin (0.76 mmol/g, Rapp Polymere, Germany). The peptide acid was prepared on Wang resin (1.25 mmol/g, Rapp Polymere, Germany). Hexadecanoic (palmitic) acid was coupled with the peptide resins using diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt). The lipopeptide was cleaved from the resin using trifluoroacetic acid (TFA) in the presence of scavengers (triisopropylsilane and water) and then precipitated in cold ether. The crude lipopeptide was purified by solid-phase extraction using aprotocol described previously [14]. The lipopeptide was eluted from the cartridge with different acetonitrile/water mixtures in the presence of 0.05% hydrochloric acid. The purity of each fraction was determined by thin layer chromatography (TLC) and reverse-phase high performance liquid chromatography (RP HPLC). The lipopeptide was characterized by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) [15].

Stock solution of FLU (Pfizer Italia S.p.A., Latina, Italy), CAS (Merck Sharp Dohme Ltd., Hoddesdon, United Kingdom) and PAL were prepared in water, while AMB (Sigma Chemical, Italy) was prepared in dimethyl sulfoxide (Sigma).

2.3. MICs and MFC determination

MICs of FLU, CAS, AMB and PAL were carried out following the Clinical Laboratory Standard (CLSI, formerly NCCLS) [28]. Drugs were used at concentration ranging from 0.125 to 64 μg/ml for FLU and PAL, and 0.03 to 16 μg/ml either for AMB and CAS. Testing was performed in RPMI 1640 medium (Sigma) buffered with 0.075 M3 (N morpholino) propanelsulfonic acid (MOPS), adjusted to pH 7.0. MICs were read spectrophotometrically at 24 and 48 h, FLU MICs was defined as the lowest drug concentration causing prominent growth reduction (approximately 80% reduction in optical density). CAS, AMB, and PAL MICs were defined as the lowest drug concentrations that prevent any discernible growth. Each isolate was tested in triplicate and *C. albicans* ATCC 90029, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750 and *C. krusei* ATCC 6258 were included in each run of the experiment as control. Minimal fungicidal concentration (MFC) was determined by streaking 100 μl from all clear MICs wells onto SDA plates. The MFC was defined as the lowest concentration of drugs that caused total inhibition of growth [31].

2.4. Synergy testing

Drug activity was assessed by a checkerboard method derived from the standardized procedure established by the CLSI. Readings were performed spectrophotometrically with an automatic plate reader (Biotek) set at 490 nm. MIC endpoints were considered the first concentration of the antifungal agent tested alone or in combination at which the turbidity in the well was 90% less than in the control well. Both on-scale and off-scale results were included in the analysis. The high off-scale MICs were converted to the next highest concentration, while the low off-scale MICs were left unchanged. Drug interactions were classified as synergistic, indifferent (Loewe additivity), or antagonistic on the basis of the fractional inhibitory concentration (FIC). FICs were calculated for MIC endpoint measurements taken from the microwell with the lowest concentration of the drug combination needed to achieve the respective endpoints. The FIC of a drug for an individual isolate was calculated as the MIC of the drug when used in combination with another drug, divided by the MIC of the drug when used alone. The FIC values were interpreted as follows: FIC $\leq$ 0.5 synergistic, FIC > 0.5 $\leq$ 4.0 indifferent and FIC > 4 antagonistic [21].

2.5. Killing curves

In killing experiments ATCC 90029, ATCC 6258, ATCC 22019 and ATCC 750 were used as reference strains. *C. albicans* 4890, *C. parapsilosis* 4796, *C. guilliermondii* 195 and *C. glabrata* 4849 (from blood and broncho alveolar lavage), *C. tropicalis* 4867 and *C. krusei* 4684 (from blood and oral swab) were included as clinical isolates. Briefly, three to five colonies of each strain from a 48 h cultures on SDA plates were suspended in sterile water and adjusted to 0.5 Mc Farland. One milliliter of the suspension was added to 9 ml of the medium RPMI-1640 with or without CAS, AMB, PAL, and incubated at 35 °C under continuous shaking at 250 cycles/min for 2 h, 4 h, 6 h and 24 h. Drugs were used at concentration of MIC, 2×, 4× and 8× MIC. At each time point, 100 μl was obtained from each solution, serially diluted, and plated on SDA plates for colony enumeration. The number of CFU was determined after incubation at 35 °C for 24–48 h. Fungicidal activity was considered to be achieved when the number of CFU/ml was <99.9% compared with the initial inoculum size [9,30].

2.6. Statistical analysis

In vitro data were compared by the Mann–Whitney test. The difference was considered to be statistically significant at $P \leq 0.05$.

3. Results

FLU MICs ranged from 0.06 to 128 μg/ml, with a MIC_{50} and a MIC_{90} of 2.0 and 64 μg/ml, respectively, AMB MICs ranged from 0.03 to1.0 μg/ml with a MIC_{50} and a MIC_{90} of 0.5 and 1.0 μg/ml, respectively, CAS MICs ranged from 0.06 to 1.0 μg/ml with a MIC_{50} and a MIC_{90} of 0.25 and 0.5 μg/ml, respectively, PAL MICs ranged from 0.5 to 16 μg/ml with a MIC_{50} and a MIC_{90} of 2.0 and 8.0 μg/ml, respectively. AMB and CAS MICs were significantly lower than those observed for either FLU or PAL ($P < 0.001$).