Peptides 30 (2009) 1794–1797

Contents lists available at ScienceDirect

Peptides

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

In vitro activity of Tachyplesin III alone and in combination with terbinafine against clinical isolates of dermatophytes

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ARTICLE INFO

Article history:
Received 17 April 2009
Received in revised form 26 June 2009
Accepted 26 June 2009
Available online 8 July 2009

Keywords:
Tachyplesin III
Terbinafine
Dermatophytes
Fungal biomass
Sinergy testing

ABSTRACT

Aim of our study was to investigate the in vitro effects of Tachyplesin III (TP), a potent disulfide-linked peptide, in dermatophytes infections, with respect to or in combination with terbinafine (TERB), against 20 clinical isolates of dermatophytes belonging to four species. A broth microdilution method following the CLSI recommendations (M38-A) was used for testing drugs alone and in combination. TERB MICs were significantly lower than those observed for TP ($p < 0.001$). Testing for antifungal agents in combination was performed for TERB with TP for all the 20 isolates. TERB activity in combination with TP showed indifferent activity for 14 of the 20 isolates (70%); synergic activity for 6 of the 20 isolates (30%); no antagonistic activity was observed. Further experiments were conducted with *Microsporum canis* 133, *Trichophyton rubrum* 62 and *Trichophyton mentagrophytes* 91 for fungal biomass. TP and TERB did not show a significant growth reduction compared to the control against *T. mentagrophytes* and *T. rubrum*. A significant difference of growth reduction both for TP and TERB compared to controls was observed for *M. canis* ($p < 0.01$). In conclusion our study demonstrated that Tachyplesin III has potential activity against dermatophytes. In addition, we observed that the in vitro activity of Tachyplesin III can be enhanced upon combination with terbinafine. Synergy could permit lower doses of the individual antifungal agents to be used more effectively and/or safely.

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1. Introduction

Several hundred antimicrobial peptides (AMPs) have been isolated from plants, insects, and vertebrates including humans, constituting host defense systems against invading pathogenic microorganisms. Accumulating evidence suggests that these peptides not only directly kill pathogens, but also modulate innate immunity and even bridge the innate and adoptive immune responses [8,9,20].

Tachyplesin III (TP; KWCFRVCYRGICYRKCR–NH_2), is a potent disulfide-linked peptide, isolated from Southeast Asian horseshoe crabs *Tachypleus gigas* and *Carcinoscorpius rotundicauda* hem lymph. The antimicrobial function appears to be related to the amphipathic structure resulting from the distribution of bulky hydrophobic groups at the "head" of a rigid antiparallel β-pleated sheet structure linked by a β-turn to a hydrophilic "tail" comprising six cationic residues derived from the N- and C-termini of the protein [10,15,18]. In previous studies Tachyplesin III has shown a broad-spectrum activity against gram-negative and -positive bacteria, enveloped viruses and yeasts and filamentous fungi [13], although there are no reports of TP activity against infections due to dermatophytes. It is well known that dermatophytoses are the result of invasion of queratinized tissues, skin, hair and nails by dermatophytes. Transmission can occur by direct contact or from exposure to desquamated cells. They cause a high morbidity, with a recent report indicating a world-wide increase of 50% in patients older than 25 years and particularly in immunocompromised patients [2,17].

In extensive dermatophytes infections or infections affecting nails or the scalp, the treatment of choice is represented by systemic antifungal treatments such as fluconazole, itraconazole and in particular terbinafine [3,5,11].

Like other allylamines, in particular terbinafine (TERB) inhibits ergosterol synthesis by inhibiting squalene epoxidase, an enzyme that is part of the fungal cell membrane synthesis pathway. Because TERB prevents convertion of squalene to lanosterol,

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0196-9781/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.peptides.2009.06.033

ergosterol cannot be synthesized. This is thought to change cell membrane permeability [11]. However there are reports about cases of remissions and relapses due to the inability of the antifungal drug to penetrate the site of infection or to the intrinsic resistance of the fungus.

The aim of this work is to evaluate, in dermatophytes infections, the activity of TP with respect to or in combination with TERB.

2. Materials and methods

2.1. Fungal strains

A total of 20 clinical isolates were tested. They included 4 strains of *Microsporum canis*, 5 of *Trichophyton mentagrophytes*, 9 of *Trichophyton rubrum* and 2 *Microsporum gypseum* collected from skin lesions, hair and nails from patients of the outpatient Clinic of Dermatology (Marche Polytechnic University, Ancona). Each isolate was identified microscopically and with biochemical tests "agar urea, agar caseina". Cultures were maintained by periodic passage on potato dextrose agar.

2.2. Antifungal agents

Tachyplesin was synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry [8]. The protected peptidyl resin was treated with the mixture: 92% trifluoroacetic acid, 2% ethanedithiol, 2% phenol, 2% water and 2% triisopropylosilane for 2 h. After cleavage the solid support was removed by filtration, and the filtrate was concentrated under reduced pressure. The cleaved peptide was precipitated with diethyl ether, dissolved in 20% acetic acid and oxidized by 0.1 M iodine in methanol. Tachyplesin was purified and analyzed by high-performance liquid chromatography (HPLC). The resulting fractions with purity greater than 94–95% were tested by HPLC. The peptide was analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). The peptide was solubilized in phosphate buffered saline (pH 7.2) yielding 1 mg/ml stock solution. Solutions were made fresh on the day of assay or stored at −80 °C in the dark for short periods.

Stock solution of TERB (Novartis-Pharma AG, Basel, Switzerland) was prepared in dimethyl sulfoxide, (Sigma, Milano, Italia), while stock solution of TP was prepared in distillated water. Further dilutions of the drugs were prepared in the test medium.

2.3. Inoculum size preparation

Dermatophyte isolates were grown on potato dextrose agar at 30 °C for 4–5 days or until good conidial growth was present. Small samples of each dermatophyte were removed from the colony surface by adding sterile saline to the plate and gently rubbing the colony surface with the tip of a Pasteur pipette. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to a sterile tube. Heavy particles were allowed to settle for 5–10 min, and the upper homogeneous suspensions were collected and filtered with sterile gauzes, mixed with a vortex mixer and observed microscopically to ensure only the presence of conidia. The densities of these suspensions were adjusted to obtain an inoculum corresponding to $2–5 \times 10^6$ CFU/ml. These stock suspensions were diluted in RPMI 1640 (Sigma) medium to obtain the final inoculum of 1×10^3 to 3×10^3 CFU/ml [12,16].

2.4. Antifungal susceptibility testing

TP and TERB MICs were determined by the microbroth dilution method following the instructions established by the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) [12]. Drugs were used at concentrations ranging from 0.06 to 64 μg/ml for TERB and from 2 to 128 μg/ml for TP. Testing was performed in RPMI 1640 medium buffered to pH 7.0 with MOPS. MICs were read at 3, 5, 7 days of incubation. The MICs were determined by visual inspection of the growth inhibition of each well compared with that of the growth control (drug-free) well. TP and TERB MICs were defined as the lowest drug concentrations preventing any discernible growth (score 0). Each isolate was tested in triplicate. *Candida parapsilosis* ATCC 22019 was included in each run of the experiment.

2.5. Fungal biomass determination

The dry weights of *M. canis* 133, *T. rubrum* 62 and *T. mentagrophytes* 91 were determined after incubation with TP and TERB. Each isolate was tested seven times in RPMI-1640 medium. Briefly an inoculum of 1×10^4 CFU/ml was added either to control tube or to the tubes containing the drugs at 2× MIC. The tubes were incubated at 32 °C under continuous shaking at 250 cycles/min for 7 days. Then, all mycelial growth was transferred into previously weighted Eppendorf tubes. After centrifugation at 14,000 for 20 min, the pellet was dried at 60 °C by using the Heto vacuum concentrator (Milano, Italy). Differential weight was determined using an analytic balance [1].

2.6. Sinergy testing

Drug activity was assessed by a checker-board method, derived from the standardized procedure established by the National Committee for Clinical Laboratory Standards [12]. Briefly, testing was performed in the same medium used for susceptibility testing. Volumes of 100 μl of each drug at a concentration of four times the targeted final concentration were dispensed in the wells of 96-well microtiter plates (Falcon 3072, Becton Dickinson). Dermatophytes inocula (50 μl of 1.0×10^3 to 5×10^3 CFU/ml) were added to each well of the microdilution trays. The trays were incubated at 35 °C and readings were performed when a prominent control growth was visualized.

Readings were performed spectrophotometrically with an automatic plate reader (Biotek) set at 490 nm. MIC endpoints were considered as 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. High off-scale MICs were converted to the next highest concentration, while low off-scale MICs were left unchanged.

Drug interactions were classified as synergistic, indifferent or antagonistic on the basis of the fractional inhibitory concentration (FIC index). The FIC index is the sum of the FICs of each of the drugs which in turn is defined as the MIC of each drug when it is used in combination divided by the MIC of the drug when it is used alone. The interaction was defined as synergistic if the FIC index was less than or equal to 0.50, indifferent if the FIC index was greater than 0.50 and less than or equal to 4.0, and antagonistic if the FIC index was greater than 4.0 [4,7].

3. Results

TP MICs for *C. parapsilosis* ATCC 22019 ranged from 2.0 to 4.0 μg/ml, TERB MICs ranged from 32 to 64 μg/ml. All clinical isolates of dermatophytes produced a detectable growth after 4 days of incubation.

TP MICs ranged from 2.0 to 64 μg/ml, TERB MICs ranged from 0.06 to 4 μg/ml.

MIC 50 and MIC 90 of TP were 16 and 64 μg/ml, respectively; IC 50 and MIC 90 of TERB were 0.25 and 2.0 μg/ml, respectively (Table 1).

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