

Human antimicrobial peptides' antifungal activity against *Aspergillus fumigatus*

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Abstract In light of the need for new antifungals, we compared the in vitro antifungal activity of two peptides derived from human lactoferrin (hLF), i.e., hLF(1–11) and hLF(21–31), two analogs of histatin 5, further referred to as dhvar4 and dhvar5, and two ubiquicidin (UBI)-derived peptides, i.e., UBI 18–35 and UBI 29–41, with that of amphotericin B against *Aspergillus fumigatus* hyphae using the MTT assay. The results revealed a dose-dependent antifungal activity for all peptides, with dhvar5 being the most potent peptide. In addition, hLF(1–11), dhvar5, and UBI 18–35 were effective against *A. fumigatus* conidia. Furthermore, hLF(1–11) did not lyse human erythrocytes, whereas dhvar5 ($\geq 16 \mu\text{M}$) and UBI 18–35 ($\geq 20 \mu\text{M}$) were hemolytic. Based on these in vitro results and their effectiveness against infections in mice, we concluded that hLF(1–11) and dhvar5 are promising candidates for the development of new agents against *A. fumigatus* infections.

Introduction

A large surveillance in the United States from 1980 through 1997 revealed that, of the deaths in which an infectious disease was the underlying cause, the mortality due to invasive mycoses increased from the tenth most common cause in 1980 to the seventh most common in 1997, with an impressive 3.6-fold increase in mortality associated with aspergillosis [1]. Furthermore, a systematic review of the literature revealed a case–fatality rate of 58% for patients with invasive aspergillosis and was especially high in bone marrow transplant recipients (87%) [2]. Moreover, amphotericin B deoxycholate and lipid formulations thereof failed to prevent death in one-half to two-thirds of these patients [2]. Despite the introduction of several new antifungals (newer generation azoles and echinocandins), the mortality and morbidity of aspergillosis is still high [2, 3].

The availability of effective strategies to improve the outcome for patients with opportunistic mycoses, in particular, invasive aspergillosis, constitutes an enormous medical need and antimicrobial peptides [4–6] may be promising candidates for this purpose because their mechanisms of action differ from that of current antifungals and the acquisition of resistant mutants is less likely to occur. An interesting approach is the use of synthetic peptides derived from natural antimicrobial proteins/peptides with antifungal activities. For example, human lactoferrin (hLF), which is a protein synthesized and released by activated neutrophils and mucosal epithelial cells, exerted antifungal activities. This antimicrobial protein contains two cationic domains in its N terminus. A recent study indicated that a synthetic peptide including the first cationic domain, further referred to as hLF(1–11), is more effective in killing *Candida albicans* than the native protein or a peptide, hLF(21–31), comprising the second cationic domain [7].

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Moreover, the hLF(1–11) peptide is effective against an invasive infection with fluconazole-resistant *C. albicans* in mice [6]. Histatin 5 is a histidine-rich, cationic peptide secreted by human parotid and submandibular glands [8]. From this peptide, several analogs with antifungal activities, further referred to as dhvar4 and dhvar5, have been designed [9]. Furthermore, our research has focused on ubiquicidin, a 6,648-Da linear cationic antimicrobial peptide produced by IFN- γ -activated murine macrophages and human airway epithelial cells [10]. This peptide displays antimicrobial activity against a variety of bacteria and *C. albicans* species. Recently, synthetic peptides derived from ubiquicidin, e.g., UBI 18–35, and UBI 29–41, have been selected on the basis of their antimicrobial activities [11]. In a first attempt to select the most promising candidates, we compared the in vitro antifungal activities of these six synthetic antimicrobial peptides against *Aspergillus fumigatus* and their hemolytic effects.

Materials and methods

Fungal strain and growth conditions

A. fumigatus (a clinical isolate) was cultured on potato dextrose agar for 4 days at 30°C. A suspension of conidia was prepared by the filtration of this culture and washed twice in 10 mM sodium phosphate buffer (NaPB; pH 7.4). In some experiments, the conidia were used as described. However, in most experiments, hyphae were studied. For this purpose, 5×10^4 colony-forming conidia ml⁻¹ of yeast nitrogen base (YNB) medium supplemented with 2% glucose were cultured in flat-bottom 24-well plates for 15 h at 30°C until >95% of the conidia had germinated.

Antimicrobial peptides

The synthetic peptides hLF(1–11) (GRRRRSVQWCA; Mw 1,374 Da), hLF(21–31) (FQWQRNMRKVR; Mw 1,567 Da), UBI 18–35 (KVAKQEKKKKKTGRAKRR; 2,169 Da), UBI 29–41 (TGRAKRRMQYNRR; 1,693 Da), and peptide 4 (part of gp120 from HIV-1; RPVVSTQLLLNGSLAEEVV; 2,171 Da) were prepared and purified as described [7]. The peptides dhvar4 (KRLFKLLFSLRKY; 1,841 Da) and dhvar5 (LLLFLKKRKRKY; 1,848 Da) were a kind gift from Dr. W. van't Hof (Academic Centre for Dentistry, Department of Oral Biochemistry, Free University, Amsterdam, the Netherlands). The purity of the various peptides ranged from 88–97%, as determined by reverse-phase high-performance liquid chromatography (RP-HPLC). The peptides were stored and used as previously described [7].

Effects of peptides against *A. fumigatus* hyphae using the MTT assay

To quantitate the antifungal activities of antimicrobial peptides, adherent hyphae were washed twice in NaPB before exposure for 1 h at 37°C to the various peptides. Peptide 4 was used as the negative control and amphotericin B as the positive control. Next, hyphae were washed twice and the mass of viable *A. fumigatus* was quantitated by a rapid colorimetric assay [12] using the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MTT). Hyphal damage was calculated by comparing the absorption by peptide-treated organisms with that from untreated organisms using the following equation:

percentage of hyphal damage

$$= \left[1 - \frac{A_{\text{with peptide/amphotericin B}}}{A_{\text{without peptide/amphotericin B}}} \right] \times 100$$

where A is the absorbance at 570 nm.

For comparison of the antifungal effects of the various antimicrobial peptides against *A. fumigatus*, we calculated the concentration of the peptides at which 50% of the maximal antifungal activity is reached by non-linear regression with the dose–effect model according to the following equation [13]:

$$E = E_{\text{max}} \times \frac{C}{EC_{50} + C}$$

where E is the observed antifungal effect at a given concentration (C) of the peptide, E_{max} is the maximal antifungal activity, and EC_{50} is the estimated concentration of the peptide at which 50% of the maximal antifungal activity is reached.

Effects of peptides against *A. fumigatus* conidia using the AlamarBlue™ assay

To compare the effects of peptides against *A. fumigatus* conidia, we determined their minimal inhibitory concentrations (MICs) as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) using AlamarBlue™ [14]. In short, 1 ml of a suspension of approximately $1-2 \times 10^3$ conidia/ml of RPMI 1640 medium with L-glutamine was pipetted together with 25 μ l of AlamarBlue™ into the wells of a 24-well plate and then the various antimicrobial peptides or, as the control, no peptide were added. After incubation for 72 h at 30°C, the color change from dark blue to red was quantitated by measuring the absorbance at 600 nm. The MIC was defined as the minimal concentration of the antimicrobial peptide preventing the development of the red color.

Hemolysis assay

We used a hemolysis assay to establish the cytotoxicity of the different peptides. Briefly, freshly collected heparinized blood from healthy individuals was centrifuged at 100g for 15 min. The erythrocytes were washed three times with PBS, centrifuged at 1,000g for 10 min, and resuspended in PBS to a concentration of 1% (v/v). Antimicrobial peptides were serially diluted in PBS (range 1.56–600 µg/ml) and 100 µl thereof was added in triplicate to 100 µl of the erythrocyte suspension. After incubation for 1 h at 37°C, the mixtures were centrifuged at 1,000g for 5 min and 150 µl of the supernatants were transferred to a flat-bottom 96-well plate for measurement of the hemoglobin release by reading the absorbance at 450 nm. PBS and 1% Tween-20 were used to establish 0% and 100% hemolysis. The percentage of intact erythrocytes was calculated using the following formula:

$$\left(1 - \frac{A_{\text{peptide}} - A_{\text{PBS}}}{A_{\text{Tween}} - A_{\text{PBS}}}\right) \times 100\%$$

where A_{peptide} is the absorbance at 450 nm in the well containing the peptide, A_{PBS} the absorbance at 450 nm in the well to which PBS was added, and A_{Tween} is the absorbance at 450 nm in the well containing Tween-20.

Statistical analyses

Differences between the values for cells exposed to antimicrobial peptides and those exposed to peptide 4 were analyzed using the Mann-Whitney *U*-test. The level of significance was set at $p < 0.05$.

Results

Antifungal activities of antimicrobial peptides

The results revealed a dose-dependent antifungal activity against *A. fumigatus* hyphae for hLF(1–11) ($R^2=0.69$; $p < 0.05$), hLF(21–31) ($R^2=0.99$; $p < 0.05$), dhvar4 ($R^2=0.83$; $p < 0.05$), dhvar5 ($R^2=0.87$; $p < 0.05$), UBI 18–35 ($R^2=0.73$; $p < 0.05$), and UBI 29–41 ($R^2=0.95$; $p < 0.05$); the control peptide 4 being ineffective (Fig. 1). The maximum hyphal damage by the various antimicrobial peptides amounted to 70–80%, which was similar to the values for ≥ 0.1 nM amphotericin B. Comparison of the EC_{50} values for the various peptides revealed that dhvar5 ($EC_{50}=3 \pm 1$ µM) is the most potent peptide; the EC_{50} values for hLF(1–11), hLF(21–31), dhvar4, UBI 18–35, and UBI 29–41 being 29 ± 5 µM, 142 ± 13 µM, 19 ± 5 µM, 37 ± 15 µM, and 91 ± 19 µM, respectively. Notably, hLF(1–11) is 5-fold more effective than hLF(21–31), dhvar5 is 6-fold more potent than dhvar4, and UBI 18–35 is almost 3-fold more effective than

UBI 29–41 in damaging *A. fumigatus* hyphae. In agreement, the MIC values for dhvar5, hLF(1–11), UBI 18–35, and amphotericin B against *A. fumigatus* conidia amounted to 3.6 ± 2.2 µM, 5 ± 4 µM, 18 ± 13 µM, and 2.2 ± 0.8 µg/ml, respectively ($n=5-7$).

Hemolytic activity of antimicrobial peptides

The peptides hLF(1–11) and UBI 29–41 did not lyse human erythrocytes at concentrations ≥ 200 µM, whereas dhvar5, hLF(21–31), and UBI 18–35 caused significant hemolysis

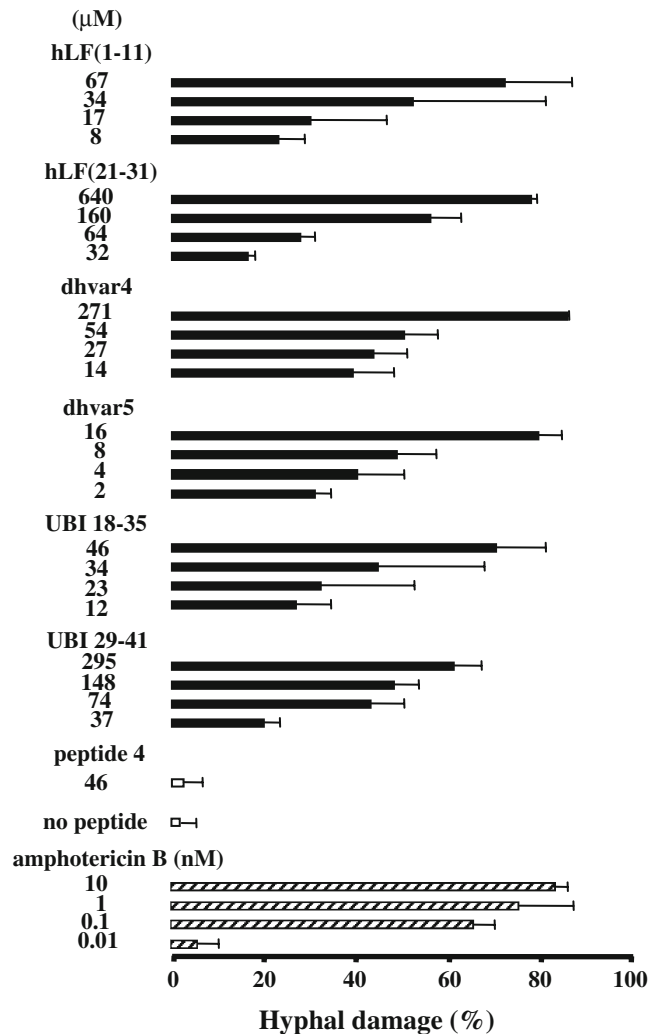


Fig. 1 Hyphal damage of *Aspergillus fumigatus* by hLF(1–11), hLF(21–31), dhvar4, dhvar5, UBI 18–35, UBI 29–41 peptides and amphotericin B. *Aspergillus fumigatus* hyphae were exposed to various concentrations of hLF(1–11), hLF(21–31), dhvar4, dhvar5, UBI 18–35, and UBI 29–41 (solid bars), peptide 4 or no peptide (open bars; negative controls) or amphotericin B (hatched bars; positive control) for 1 h. Next, the mass of viable *A. fumigatus* was quantitated by the MTT assay. Hyphal damage was calculated by comparing the absorbance at 570 nm by peptide-treated organisms with that by control organisms either untreated or treated with peptide 4. The results are shown as the means (plus standard deviation [SD]) of at least three independent experiments

(>2–3%) at $\geq 16 \mu\text{M}$, $\geq 16 \mu\text{M}$, and $\geq 20 \mu\text{M}$, respectively. The hemolytic activity of dhvar4 was not tested.

Discussion

The main conclusion from the present results is that hLF(1–11) and dhvar5 are the most promising candidates for the development of new agents to treat infections with *A. fumigatus*. This conclusion is based on the following lines of evidence.

First, dhvar5 and hLF(1–11) were among the most potent peptides against *A. fumigatus* hyphae in vitro, and they were also active against *A. fumigatus* conidia in vitro. It should be realized that the antifungal activities of antimicrobial peptides in vitro may not reflect their antifungal activity in infected animals. For example, we reported that the doses of hLF(1–11) required for antifungal effects in mice with a disseminated *C. albicans* infection were much lower than that required in in vitro experiments [6]. Possible explanations for these observations include synergistic/additive effects between this peptide and host-derived antifungal factors, such as endogenous antimicrobial proteins/peptides and reactive oxygen intermediates [6]. Furthermore, the hLF(1–11) peptide, and perhaps dhvar5 as well, stimulates the antimicrobial activities of host cells, e.g., human mononuclear phagocytes [15]. Second, at the highest concentrations tested, no toxicity of hLF(1–11) and UBI 29–41 on human erythrocytes has been observed, while dhvar5 and hLF(21–31) caused significant hemolysis at a concentration $\geq 16 \mu\text{M}$ and UBI 18–35 at a concentration $\geq 20 \mu\text{M}$. Moreover, safety studies with hLF(1–11) in laboratory animals and humans have been successfully completed. Thirdly, all peptides under investigation displayed antibacterial activities against infections with, e.g., methicillin-resistant *Staphylococcus aureus* (MRSA) in mice [11] and the hLF(1–11) peptide is effective against an invasive *C. albicans* infection in mice [6]. Since both hLF(1–11) and dhvar5 act synergistically in vitro with current antifungal agents against *Candida* species [9, 16], it may be expected that such combinations are also highly effective against infections with *A. fumigatus*. If effective in vivo, such combinations may reduce the dose and duration of anti-infective agents and the use of multiple antibiotics, thereby, limiting the emergence of drug-resistant strains.

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