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Fungicidal activity of human lactoferrin-derived peptides based on the antimicrobial $\alpha\beta$ region

N. Kondori ^a, L. Baltzer ^b, G.T. Dolphin ^c, I. Mattsby-Baltzer ^{a,*}

^a *Department of Infectious Medicine/Clinical Bacteriology, University of Gothenburg, Guldhedsgatan 10, S-413 46 Gothenburg, Sweden*

^b *Department of Biochemistry and Organic Chemistry, Uppsala University, S-751 24, Uppsala, Sweden*

^c *Department of Chemistry, Linköping University, 581 83 Linköping, Sweden*

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* Corresponding author. Present address: Department of Clinical Bacteriology, Göteborg University, Guldhedsgatan 10, S-413 46 Göteborg, Sweden. Tel.: +46 31 342 47 28; fax: +46 31 342 49 75.

E-mail address: inger.mattsby-baltzer@microbio.gu.se (I. Mattsby-Baltzer).

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ABSTRACT

Owing to the increasing number of infections in hospitalised patients caused by resistant strains of fungi, there is a need to develop new therapeutic agents for these infections. Naturally occurring antimicrobial peptides may constitute models for developing such agents. A modified peptide sequence (CFQWKRAMRKVR; HLOpt2) based on amino acid residues 20–31 of the *N*-terminal end of human lactoferrin (hLF) as well as a double-sized human lactoferricin-like peptide (amino acid residues 16–40; HLBD1) were investigated for their antifungal activities in vitro and in vivo. By in vitro assay, HLOpt2 was fungicidal at concentrations of 12.5–25 µg/mL against *Cryptococcus neoformans*, *Candida albicans*, *Candida krusei*, *Candida kefyr* and *Candida parapsilosis*, but not against *Candida glabrata*. HLOpt2 was demonstrated to have ≥ 16 -fold greater killing activity than HLBD1. By inducing some helical formation caused by lactam bridges or by extending the assay time (from 2 h to 20 h), HLBD1 became almost comparable with HLOpt2 in its fungicidal activity. Killing of *C. albicans* yeast cells by HLOpt2 was rapid and was accompanied by cytoplasmic and mitochondrial membrane permeabilisation as well as formation of deep pits on the yeast cell surface. In a murine *C. albicans* skin infection model, atopic treatment with the peptides resulted in significantly reduced yields of *Candida* from the infected skin areas. The antifungal activities of HLOpt2 in vitro and in vivo suggest possible potential as a therapeutic agent against most *Candida* spp. and *C. neoformans*. The greatly improved antifungal effect of the lactam-modified HLBD1 indicates the importance of amphipathic helix formation for lethal activity.

1. INTRODUCTION

Candida albicans is an opportunistic pathogen that causes mucosal and systemic infections in immunosuppressed patients. Because of the rising number of failures in the treatment of mycoses in severely immunosuppressed individuals owing to resistant strains of *Candida*, there is a need to develop new therapeutic agents that either on their own exert antifungal activity or in combination with conventional antimycotics enhance their activity.

Lactoferrin (LF) is a single-chain, iron-binding glycoprotein found in milk, mucosal secretions and the secondary granules of neutrophils. It is an innate defence factor expressing antimicrobial, anti-inflammatory and immunoregulatory activities [1–3]. Pepsin digestion of LF releases a fragment, lactoferricin, which is several times more bactericidal than the parent molecule [4,5]. Human lactoferricin consists of amino acid residues 1–46 from the *N*-terminal end of LF, including two disulphide bonds [4,6].

Amino acid residues 14–31 form an α -helix in the human LF (hLF) molecule, of which the latter half is amphipathic [7,8]; antibacterial activity resides in this part. A short peptide fragment comprising amino acid residues 21–31 was shown to retain bactericidal activity, but was less active against *C. albicans* than a peptide fragment containing amino acid residues 1–11 from the *N*-terminal end of hLF [8–10].

In an earlier study on synthetic peptide fragments derived from the antibacterial α -helix region of hLF, it was found that peptides comprising amino acid residues 17–31, 18–31, 19–31 and 20–31 from the *N*-terminal end expressed stronger microbicidal activity than the peptide fragment 21–31 [11]. By changing two neutral amino acid

residues in the natural peptide sequence 20–31 (HL10) to one charged (Lys) and one hydrophobic (Ala) amino acid (forming HLOpt2), fungicidal activity was enhanced.

The aim of this study was to explore the antifungal activity of HLOpt2 against *Candida* spp. and *Cryptococcus neoformans* and to compare its activities with the double-sized lactoferricin-like synthetic fragment HLBD1 (amino acids 16–40). In addition, the antifungal effects of these two peptides were investigated in an experimental *C. albicans* skin infection model.

2. Materials and methods

2.1. Fungal strains

Three strains of *C. albicans* (ATCC 64549, CCUG 90008 and CCUG 5594), three strains of *Candida glabrata* (ATCC 90030, CCUG 85247 and CCUG 42987), three strains of *Candida parapsilosis* (ATCC 22019, CCUG 37233 and patient isolate *C. parapsilosis* Ir1), one strain of *Candida krusei* (ATCC 6258), one patient isolate of *Candida kefyr* Ir2 and two strains of *C. neoformans* (CCUG 37833 and patient isolate *C. neoformans* Ir3) were studied. *Candida albicans* ATCC 64549 was used as the main test organism for the fungicidal activities of the peptides. For susceptibility tests, all strains were cultivated in brain–heart infusion (BHI) medium at 37 °C overnight and part of the culture was transferred to fresh BHI medium for another 2 h of growth. Conversion of yeast to germ tube and hyphal forms of *C. albicans* was carried out by transferring the *C. albicans* yeast cells to RPMI 1640 followed by cultivation at 37 °C for 24 h.

2.2. Lactoferrin and peptides

LF from human milk was purchased from Sigma (St Louis, MO). hLF peptides were synthesised using a 9-fluoroyl-mehoxycarbonyl continuous-flow strategy on a Pioneer™ automated peptide synthesizer (Applied Biosystems, Foster City, CA) and have been described previously [11].

2.2.1. Synthesis of lactam-bridged HLBD1

Lactam bridges were introduced into HLBD1 by changing the amino acids to Glu (E) and Lys (K) four amino acids apart (Fig. 1). Lactam formation between the side chains was achieved by treatment with a six-fold excess of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphoniumhexafluorophosphate-*N*-hydroxybenzotriazole-diisopropylethylamine (PyBOP-HOBt-DIPEA) (1:1:2) in dimethylformamide (DMF) for 16 h. The resin was washed with DMF and dichloromethane and was dried under vacuum. The peptide was cleaved from the resin as described previously [11]. Lactam bridge formation induces some helical content in short peptide fragments that are random coiled. Helix formation is readily detected by circular dichroism (CD) spectroscopy, and increased helical content upon lactam bridge formation was concluded from the increased negative value of the mean residue ellipticity at 208 nm and 222 nm.

The peptide sequences used in this study are shown in Fig. 1.

2.3. Antifungal drugs

Amphotericin B (AmB) and flucytosine (5-FC) were purchased from Sigma. Fluconazole (FLZ) was obtained from Pfizer (Illertissen, Germany). FLZ and 5-FC were dissolved in sterile water and physiological saline (1 mg/mL), respectively, whereas AmB was first dissolved in dimethyl sulphoxide (DMSO) (20 mg/mL) before diluting it further.

2.4. Minimum microbicidal concentrations (MMCs) and minimum inhibitory concentrations (MICs)

The microplate assay has been described elsewhere [11]. In short, peptides were serially diluted in 0.03% BHI (BHI_{dil}) or 1% Bacto™ Peptone (BP) in two-fold steps and were incubated with yeast cells at a concentration of 2×10^5 cells/mL for 2 h at 37 °C. Viability of the inoculum was analysed by culturing 5 µL of the suspension of each well on blood agar plates for 20 h at 37 °C. The concentration of hLF or peptide corresponding to the dilution at which ≥ 99.9 % killing of the inoculum was obtained was defined as the MMC value. A four-fold difference in MMC values (two-fold titre steps) was regarded as significantly different. In some experiments, the incubation time of the microplate was extended to 20 h.

Moreover, in some experiments the microplate was incubated for 20–44 h at 37 °C in order to analyse MIC values of the peptides or hLF. The MIC value was defined as the concentration of hLF or peptide corresponding to the dilution at which the absorbance at 650 nm was reduced to at least 50% of that of the untreated inoculum. Absorbance was read in an EMax® Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.5. *Candida* membrane permeability

The DNA-binding fluorescent probe propidium iodide (PI) (Sigma) was used to analyse changes in membrane permeability of *Candida* upon exposure to hLF or peptides. A fluorescence-activated cell sorter (FACS) was used to monitor permeability changes. Briefly, yeast cells were grown overnight and were then transferred to new BHI medium for another 2 h of growth.

Cells were washed and diluted in 10 mM phosphate buffer (pH 7.2). Yeast cells plus hLF or peptide at various concentrations were incubated for 2 h at 37 °C. The final concentration of yeast cells was 2×10^6 cells/mL. The cells were then re-incubated at room temperature with 1 µg of PI for 5 min followed by analysis on a FACScan Flow Cytometer (BD Biosciences, San José, CA).

2.6. *Candida* mitochondrial membrane permeability

The fluorescent probe rhodamine 123 (Sigma) was used to study mitochondrial permeability [12] since it accumulates in this cell compartment. In short, *C. albicans* in log phase was suspended in potassium phosphate buffer (1 mM, pH 7.0) and was incubated with rhodamine 123 (5 µM) for 15 min at 37 °C in a water-bath. After 3 washes with K-phosphate buffer, the cells were treated for 10 min at 37 °C with hLF or peptide at final concentrations of 2000 µg/mL and 25 µg/mL, respectively. The distribution of rhodamine 123 in *Candida* cells was examined using a fluorescence microscope (Leica Microsystems Cambridge Ltd., Cambridge, UK).

2.7. Electron microscopy

Candida albicans ATCC 64549 and *C. neoformans* CCUG 37833 were incubated overnight. Fresh BHI medium was inoculated with the cultures, which then was incubated for another 2 h at 37 °C. After washing, cells were suspended in 10 mM phosphate buffer at a final concentration of 2×10^7 cells/mL. hLF or HLopt2 at final concentrations of 4000 µg/mL and 200 µg/mL, respectively, was added to the cell suspension and was incubated for 2 h at 37 °C. High concentrations of hLF and the peptide were chosen in order to achieve killing of a high number of yeast cells. Cells were separated from the suspensions by centrifugation and were fixed with an aldehyde mixture (2% paraformaldehyde, 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2) diluted 1:1 with phosphate-buffered saline (PBS). The suspension was then washed with PBS.

Yeast cells (250 µL) were transferred to poly-L-lysine-treated, gold-coated Thermanox™ coverslips (Nunc A/S, Roskilde, Denmark) for 30 min to allow the cells to adhere to the surface. After rinsing with PBS, cells were prepared for scanning electron microscopy (SEM) using the OTOTO method comprising repeated treatments with osmium tetroxide and thiocarobhydrazide [13]. The coverslips were dehydrated in ethanol and were infiltrated with hexamethyldisilazane, which was allowed to evaporate in a fume hood. The dried specimens were mounted on aluminium SEM stubs and were examined without thin film metal coating in a Zeiss 982 Gemini scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

2.8. Haemolysis

Human red blood cells were washed and diluted in PBS to a concentration of 2%. A volume of 25 μL of the erythrocyte suspension was added to the wells of a microplate. Peptides and AmB solutions were diluted in two-fold steps (25–100 $\mu\text{g}/\text{mL}$) and were added in 50 μL volumes to the wells. All concentrations were run in triplicate. The microplate was incubated for 1 h at 37 °C. The optical density at 650 nm was then measured in a microplate reader and was compared with the detergent hexadecyltrimethylammonium bromide (CTAB) (Sigma) that is known to lyse erythrocytes.

2.9. Anticandidal activity of HLOpt2 and HLBD1 in vivo

2.9.1. Cutaneous candidiasis

Candida albicans ATCC 64549 was cultured overnight in Sabouraud broth at 37 °C. Yeast cells were then washed and diluted in phosphate buffer (10 mM, pH 7.4) to 2×10^{10} cells/mL. BALB/c mice (8–10 weeks old) were treated with *C. albicans* on a shaved area by gently rubbing a volume of 10 μL (2×10^8 yeast cells per 12-mm diameter spot) with a blunt plastic rod during isoflurane anaesthesia [14]. The next day, anaesthetised mice were treated with 10 μL of a peptide solution or vehicle (phosphate buffer) by applying the volume onto the infected spots. After 4 h the treatment was repeated. Animals were killed the following day. Viable counts were determined from washings obtained from the treated areas using a metal ring placed over the spot and gentle rubbing in the presence of 70 μL of phosphate buffer containing Triton X-100 (0.01%). The procedure was repeated once giving a total sample volume of ca. 100 μL for culturing.

3. Results

3.1. Antifungal activity of HLOpt2, HL10 and HLBD1

3.1.1. Comparison with conventional antimycotics

The modified 12-amino acid long peptide HLOpt2 and its natural counterpart HL10 as well as the double-sized lactoferricin-like HLBD1 (Fig. 1) were compared with the parental hLF molecule as well as with several conventional antimycotics using BHI_{dil} as medium in the antifungal microplate assay (Table 1). All peptides had significantly greater fungicidal activity than hLF. Comparison with AmB showed that HLOpt2 was in the same range as this antimycotic, whilst all three peptides had stronger fungicidal activity in comparison with 5-FC and FLZ. Regarding growth inhibition, only AmB expressed a significantly lower MIC value compared with its MMC value.

In line with hLF, the presence of pseudohyphae did not alter the antifungal activity of one tested peptide (HLBD1) against *C. albicans* ATCC 64549 after 2 h of incubation compared with yeast form (data not shown).

3.1.2. Kinetics of the fungicidal activity of HLOpt2 and HLBD1

The antimicrobial activity of cationic peptides is strongly influenced by the assay broth used. Since earlier studies had shown that HLBD1 and hLF expressed significantly reduced fungicidal activity in the 2-h microplate assay when replacing BHI_{dil} with BP, we investigated whether the difference in fungicidal activity was due to a slower killing rate in the presence of BP medium. Thus, the fungicidal activity of HLOpt2, HLBD1

and hLF was followed over 1 min to 20 h of incubation in BHI_{dil} and was compared with that in BP.

In BHI_{dil} the shortest peptide HLopt2 reduced the number of yeast cells to 5% of the inoculum within the first minute, whilst no yeast cells could be detected at 5 min (Fig. 2). It took 1 h for HLBD1 and 2 h for hLF to reduce the number of cells below the detection limit (99.9%). However, in BP the corresponding time for $\geq 99.9\%$ killing for HLopt2 and HLBD1 was 30 min and 20 h, respectively, whilst no killing was observed with hLF.

3.1.3. Fungicidal activity against *Candida* spp. and *Cryptococcus neoformans*

Changing the culture medium to 1% BP affected the killing capacity of HLBD1 and HL10 dramatically (Table 2). Thus, MMC values for *C. albicans* ATCC 64549 increased 16-fold for HLBD1 and 8-fold for HL10 in order to achieve the same killing activity as in BHI_{dil} (Table 1). However, the activity of HLopt2 did not change significantly. Furthermore, HLopt2 had significantly greater fungicidal activity compared with the other two peptides and hLF against all *Candida* and *C. neoformans*, except for *C. glabrata* (Table 2) (HLopt2 vs. HL10, $P = 0.0020$, Wilcoxon signed rank test). Neither the peptides nor hLF killed *C. glabrata* at the highest concentration tested.

3.1.4. Effects of some preformed helical content in HLBD1

The fungicidal activity of HLBD1 was dramatically reduced in BP medium (Table 1 and 2; Fig. 2). Thus, it was investigated whether any preformed helical content in HLBD1

would improve its fungicidal activity in BP. According to the results of a previous report [15], positions 22, 26 and 30, in addition to position 18 (Thr), were suitable for lactam bridges (Fig. 1). The lactam bridges induced some helical content as confirmed by CD spectroscopy. In contrast to HLBD1 lactam bridge 18–22, lactam bridges between positions 22–26 and 26–30 lowered the MMC values significantly against *C. albicans* ATCC 64549 at 2 h compared with the native sequence of HLBD1 (Table 3). The activities of the two lactam-bridged HLBD1 peptides were comparable to HLOpt2 (Table 2). After 20 h of incubation, MMC values of HLBD1 lactam bridges 22–26 and 26–30 against *C. albicans* did not change from those at 2 h, whilst MMC values for HLBD1 lactam bridge 18–22 and HLBD1 were significantly lower (Table 3).

3.2. Permeabilisation of fungal membranes by HLOpt2 and HLBD1

Permeabilisation of the cytoplasmic membrane of *C. albicans* ATCC 64549 induced by the peptides after 2 h of incubation was demonstrated by the percentage of PI-positive cells (Fig. 3). The percentage of PI-positive cells, which ranged between 96% and 98% at 20 $\mu\text{g}/\text{mL}$ of peptides, reflected the killing shown by the MMC assay. Thus, killing at 20 $\mu\text{g}/\text{mL}$ was $\geq 99.9\%$ by the MMC assay except for hLF (data not shown). hLF at 4 mg/mL gave rise to 16% PI-positive cells (Fig. 3), whilst no reduction was recorded by the MMC assay (data not shown).

Rhodamine 123 fluorescence was found localised to subcellular compartments presumed to be mitochondria in the yeast cells treated with buffer (data not shown). HLOpt2 changed this pattern of fluorescence in the cells by showing an even distribution all over the cell. hLF-treated cells remained unaffected after 10 min of

incubation. This result was expected since no fungicidal effect of hLF on *C. albicans* ATCC 64549 could be recorded at this time point (Fig. 2).

The effects of HLOpt2 and hLF on the cell wall of *C. albicans* and *C. neoformans* were studied by SEM using 10 mM phosphate buffer as incubation medium. HLOpt2 treatment changed the surface of the yeasts greatly (Fig. 4). The *C. albicans* surface appeared punctured. Deep pits were seen all over the surface in combination with scattered blebs or knobs. An even rougher surface was observed for *C. neoformans* in addition to leakage of material between the cells. In addition to the pits, the surface was heavily covered with blebs or knobs (Fig. 4). More than 99.9% of the yeast cells were dead following incubation with HLOpt2.

After 2 h of incubation of the yeast cells in the presence of hLF, the cells were agglutinated and cell surface alterations were observed. A more ruffled cell surface was noticed compared with untreated cells (Fig. 4). Killing activity of hLF during this incubation period was not observed as determined by the antifungal assay (data not shown).

3.3. Membrane damaging activity on erythrocytes

The membrane destabilising effect of HLBD1, HLOpt2 and AmB was assessed by haemolysis of human red blood cells (Fig. 5). The peptides induced ca. 8% haemolysis at 100 $\mu\text{g/mL}$, whilst no further significant increase in haemolysis was recorded at the highest concentration tested (200 $\mu\text{g/mL}$). The 50% haemolysis value of the membrane-damaging detergent CTAB was 37 $\mu\text{g/mL}$.

3.4. Anticandidal activity of HLOpt2 and HLBD1 in vivo

In an experimental fungal infection, mice were treated twice with 100 µg or 400 µg of peptide per spot. A significant reduction in colony-forming units in the washings from the infected area was achieved with both peptides at a dose of 400 µg/spot (Table 4). No significant reduction was observed with the lower dose.

4. Discussion

In a previous report on the structure–antimicrobial activity relationship of hLF-derived peptides, the modified peptide HLOpt2 comprising 12 amino acids (Fig. 1) expressed the strongest antimicrobial activity against the three test organisms *Escherichia coli*, *Staphylococcus aureus* and *C. albicans* [11]. In the present study, the antifungal activity of HLOpt2 was further explored. HLOpt2 was shown to be fungicidal against *C. neoformans*, *C. albicans*, *C. parapsilosis*, *C. kefyr* and *C. krusei*, but not against *C. glabrata*.

The enhanced antifungal activity of HLOpt2 (4–8-fold) compared with its natural counterpart was due to additional charged and hydrophobic amino acids, substituting the neutral hydrophilic amino acid residues Asp and Glu in the natural peptide sequence of HL10 [11]. Moreover, HLOpt2 was more than 100-fold more active (>3-fold on a molar basis) against several *Candida* strains and *C. neoformans* compared with intact hLF, which is in line with an earlier report on lactoferricin and hLF [4].

Although the lactoferricin-like and double-sized HLBD1 was less effective than HLOpt2, induction of some helical content by lactam bridge formation (positions 22–26 or 26–30) (Fig. 1) in the part of the molecule that corresponds to the amphipathic half

of the α -helix of hLF promoted a more rapid killing of *C. albicans*, approaching the MMC value of HLOpt2.

However, after 20 h of incubation the MMC values were almost identical for HLBD1 with or without lactam bridges. One explanation for the enhancing effect of some helical content may be that pre-organisation of part of an amphiphilic conformation could speed up the binding processes of the peptide to targets on the fungal membrane. Recently, human lactoferricin was suggested to display a nascent helix from residue 14 to 29 in aqueous solution as a result of the hydrophobic side chains [16].

The different killing kinetics of *C. albicans* by the peptides and hLF in the two nutrient broths indicated possibly increased concentrations of metallic salts in 1% BP compared with 0.03% BHI. In a previous report on the sensitivity of HLOpt2 to metallic salts, particularly Ca^{2+} , concentrations of 2.5 mM reduced its candidacidal activity [11].

In this study, HLOpt2 appeared to have induced both large openings/pits in the outer part of the yeast cell wall of *C. albicans* as well as loss of membrane integrity as indicated by SEM and PI staining, respectively [17]. Furthermore, loss of mitochondrial membrane integrity suggests that the peptide also accumulated in the cytoplasm and targeted intracellular organelles. Thus, the effects of HLOpt2 resembled the direct interaction of bovine lactoferricin with the cell surface of *C. albicans* as well as the membrane permeabilising activity of the human lactoferricin-like peptide sequence 18–40 on *C. albicans* cells [18,19].

In general, the antifungal mechanism of antimicrobial peptides appears to involve rupture of the fungal membrane and suppression of mitochondrial respiration [3,20]. However, less is known about the targets for peptide binding in the cytoplasmic membrane and the following events of the killing mechanism.

The synthetic peptide fragment comprising amino acid residues 1–11 from the *N*-terminal end of the hLF molecule (hLF11) was suggested to interact with structural elements of the plasma membrane of *C. albicans* and to be taken up by an energy-dependent way. Thus, candidacidal activity was dependent on mitochondrial respiration and a subsequent rise in extracellular ATP [10]. Moreover, the hLF11-induced PI uptake was associated with non-lytic ATP release of *C. albicans*. A bovine lactoferricin-derived peptide comprising amino acid residues 12–21 was shown to require the Trk1p potassium transporter for fungicidal activity, but not the additional cell wall proteins ssA1/2p (heat shock 70 family) as shown for histatin 5 [21].

The reason for the lack of effect of the peptides against *C. glabrata* is not known. *Candida glabrata* has repeatedly been identified as being the least sensitive among the *Candida* spp. to many azoles. Helmerhorst et al. [22] showed that *C. glabrata* is also insensitive to several cationic antifungal proteins. Investigation of the resistance of *C. glabrata* to histatin 5 suggested that the cellular characteristics providing resistance to azoles were distinct from those of cationic antifungal peptides [15,23,24].

Comparison of HLBD1 and HLopt2 with conventional antimycotics such as 5-FC, FLZ and AmB showed that the peptides were comparable with AmB in their fungicidal activity. This is to be expected since AmB also causes impairment of membrane barrier function and cell death, in contrast to the other two antimycotics that are

fungistatic. The mechanism of action of AmB involves binding to ergosterol, which may explain why AmB compared with HLOpt2 and HLBD1 has a somewhat weaker haemolysis effect than the peptides.

To prevent candidal infection in immunosuppressed patients, colonisation with *Candida* spp. should be avoided. However, preventive treatment with conventional antimycotics is not recommended owing to resistance development. The risk is lower with naturally derived charged peptides since these rarely induce microbial resistance [25]. In the present study, an experimental model of cutaneous *Candida* infection was used to investigate the antifungal effects of HLOpt2 and HLBD1. Both peptides showed protective activities by significantly reducing the number of *C. albicans* recovered from the infected skin area. These results agree with the earlier reported anti-infectious activity of HLBD1 in *E. coli*-induced urinary tract infection in mice [26]. Whether the antifungal activity of HLOpt2 was superior to HLBD1 in vivo could not be ruled out in this study.

In summary, HLOpt2, originally based on a part of the antimicrobial α -helix region of the hLF molecule, exerts fungicidal activity against several *Candida* spp. as well as *C. neoformans*. The antifungal effect is suggested to involve disarrangement of the cell wall and a mode of action that results in disruption of the cytoplasmic and mitochondrial membranes. By introducing some pre-organised helix conformation into the double-sized HLBD1, a killing activity almost comparable with that of HLOpt2 could be observed in vitro. The antifungal effect of HLOpt2 against experimental *Candida* skin infection indicates a potential therapeutic use.

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Competing interests

None declared.

Ethical approval

Ethical approval was received from the local animal ethical committee in Gothenburg, Sweden (no. 150-2001).

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Fig. 1. Peptides derived from the antimicrobial region of human lactoferrin (hLF). HLBD1 was capped at the *N*-(ac) and *C*-(amide) termini. The sequence of peptide HL10 was modified by changing the amino acid residues at positions 24 and 26 to the amino acids shown in bold letters to form HLOpt2.

Fig. 2. Killing kinetics of the peptides HLBD1 (200 µg/mL) and HLOpt2 (100 µg/mL) compared with human lactoferrin (hLF) (2000 µg/mL). Peptides and human LF were incubated with *Candida albicans* in 0.03% brain–heart infusion (BHI_{dil}) or 1% Bacto™ Peptone (BP) for 20 h. Samples were collected directly after mixing and at 5, 10, 30, 60 and 120 min. Each point represents the mean of duplicates of colony-forming units (CFU)/mL (10 log) of the inoculum remaining alive. The broken line shows 99.9% reduction of the inoculum.

Fig. 3. Propidium iodide (PI)-staining of *Candida albicans* incubated for 2 h at 37 °C with various concentrations of the peptides HLBD1, HL10 and HLOpt2 (1, 20, 100 and 500 µg/mL) or human lactoferrin (hLF) (1, 200, 1000 and 4000 µg/mL). Uptake of PI was recorded by FACScan analysis and the percentage of PI-positive cells was calculated.

Fig. 4. Ultrastructural features of *Candida albicans* and *Cryptococcus neoformans* exposed to 4000 µg/mL human lactoferrin (hLF), 200 µg/mL HLOpt2 or buffer alone (control) for 2 h (magnification ×10 000 and ×50 000).

Fig. 5. Haemolysis in the presence of peptides (HLOpt2) and HLBD1), amphotericin B or the detergent hexadecyltrimethylammonium bromide (CTAB). Erythrocytes and the

various agents were incubated for 1 h. Haemolysis was measured spectrophotometrically. Each point represents the mean of triplicates and the bars show the standard error of the mean.

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Table 1

Fungicidal and fungistatic activity of the peptides HLBD1, HL10 and Hlopt2 as well as human lactoferrin (hLF) and conventional antimycotics against *Candida albicans* ATCC 64549 in BHI_{dil}. Two-fold serial dilutions starting from 200 µg/mL of peptide or antimycotic and from 2000 µg/mL of hLF were used in the assay ^a

Peptide	MMC (µg/mL) ^b	MIC (µg/mL)
HLBD1	12.5	6.2
HL10	12.5	12.5
Hlopt2	6.2	N/D ^α
hLF	1000	500
AmB	3.1	0.4
Flucytosine	>200	200
Fluconazole	>200	≥200

BHI_{dil}, 0.03% brain-heart infusion; MMC, minimum microbicidal concentration; MIC, minimum inhibitory concentration; AmB, amphotericin B; N/D, not determined.

^a All samples were analysed in triplicate in two separate experiments.

^b The MMC values of the two experiments were identical.

Table 2

Fungicidal effects of the peptides HLBD1, HL10 and HLOpt2 compared with human lactoferrin (hLF) in 1% Bacto™ Peptone. Two-fold serial dilutions starting from 2000 µg/mL for hLF and 400 µg/mL for the peptides were used

Organism	MMC (µg/mL) ^a			
	hLF	HL10	HLOpt2	HLBD1
<i>Candida albicans</i>				
ATCC 64549	>2000	100	12	>200
CCUG 90008	>2000	200	25	N/D
CCUG 5594	>2000	100	25	N/D
<i>Candida glabrata</i>				
ATCC 90030	>2000	>400	>400	N/D
CCUG 85247	>2000	>400	>400	N/D
CCUG 42987	>2000	>400	>400	N/D
<i>Candida parapsilosis</i>				
Ir1 ^b	>2000	100	25	N/D
CCUG 37233	>2000	200	25	N/D
CCUG 22019	>2000	100	12	N/D
<i>Candida kefyr</i>				
Ir2 ^b	>2000	100	12	N/D
<i>Candida krusei</i>				
CCUG 6258	>2000	100	12	N/D
<i>Cryptococcus neoformans</i>				
Ir3 ^b	>2000	50	12	N/D
CCUG 37833	>2000	50	12	N/D

MMC, minimum microbicidal concentration; N/D, not determined.

^a All samples were run in duplicate, giving identical MMC values.

^b Patient isolate.

Table 3

Effect on microbicidal activity of some helical content in HLBD1 caused by the introduction of a lactam bridge. *Candida albicans* ATCC 64549 was incubated with the peptides in 1% Bacto™ Peptone and killing activity was assessed at 2 h and 20 h. Peptides were serially diluted in two-fold steps starting at 200 µg/mL

Substitution of amino acid residue for lactam bridge formation	MMC (µg/mL) ^a	
	2 h	20 h
HLBD1 lactam bridge		
18–22	>200	50
22–26	25	25
26–30	25	25
HLBD1	>200	50

^a All samples were run in duplicate, giving identical MMC values.

Table 4

Experimental skin infection by *Candida albicans* ATCC 64549 in mice. Peptide (400 $\mu\text{g}/\text{spot}$) or vehicle alone (phosphate buffer) was applied twice, 4 h apart, onto a shaved area on the back of animals infected with *C. albicans* 24 h previously. The next day viable counts of washings from each spot were determined by culturing on Sabouraud plates

Peptide	No. of experiments	CFU (mean \pm S.E.)		<i>P</i> -value ^a
		Peptide treatment	Vehicle alone	
HLBD1	2	191 \pm 57	414 \pm 60	0.0148
HLopt2	2	160 \pm 60	570 \pm 251	0.0070

S.E., standard error.

^a Mann–Whitney.

Peptide code	Sequence
HLBD1 (Mw 3057 g/mol)	<div style="text-align: center;">16 20 25 30 35 40</div> ac-EATKCFQWQRNMRKVRGPPVSCI K R-amide
Lactam bridges:	
HLBD1(18-22) (Mw 3069 g/mol):	
HLBD1(22-26) (Mw 3056 g/mol)	
HLBD1(26-30) (Mw 3085 g/mol)	
HL10 (Mw 1709)	CFQWQRNMRKVR
HLopt2 (Mw 1609)	CFQWKRAMRKVR

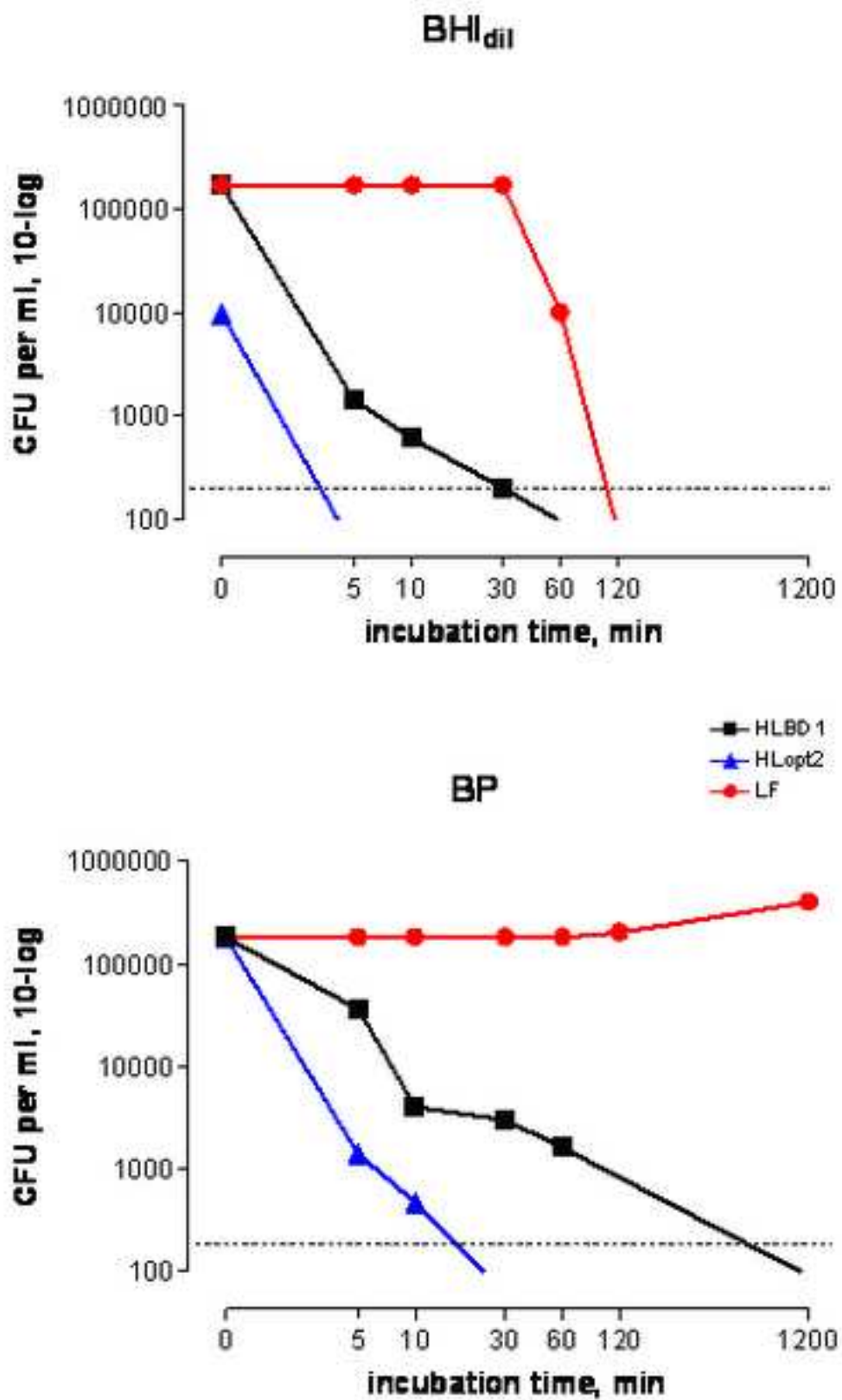


Fig 1

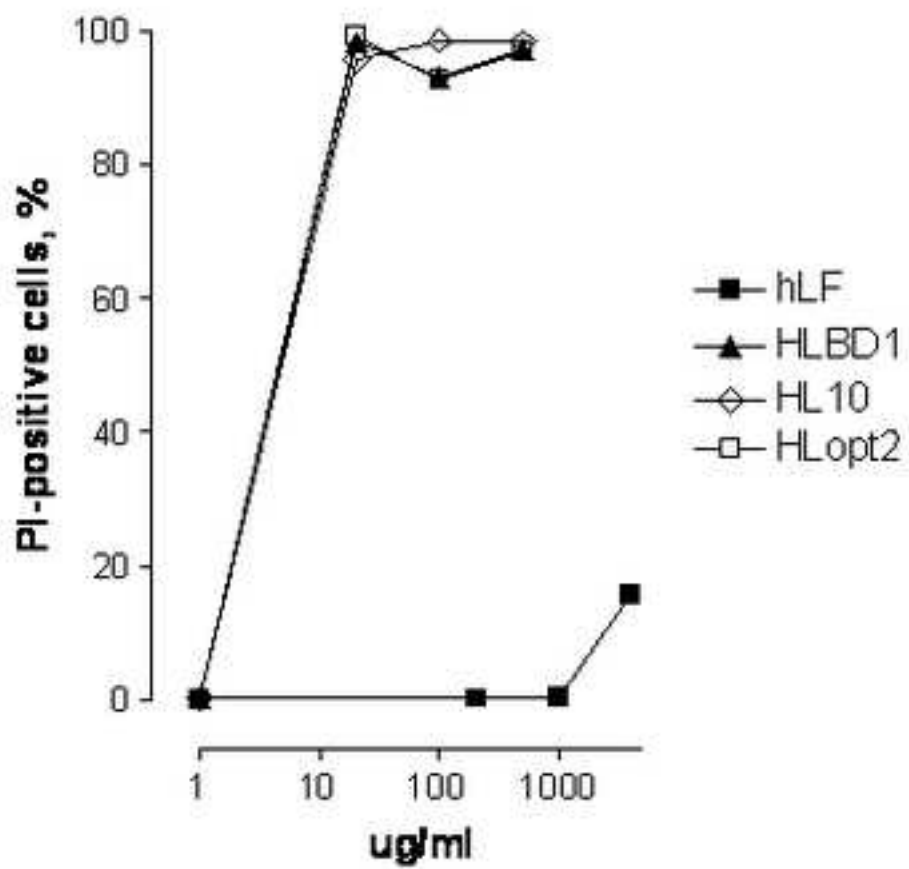


Fig 2

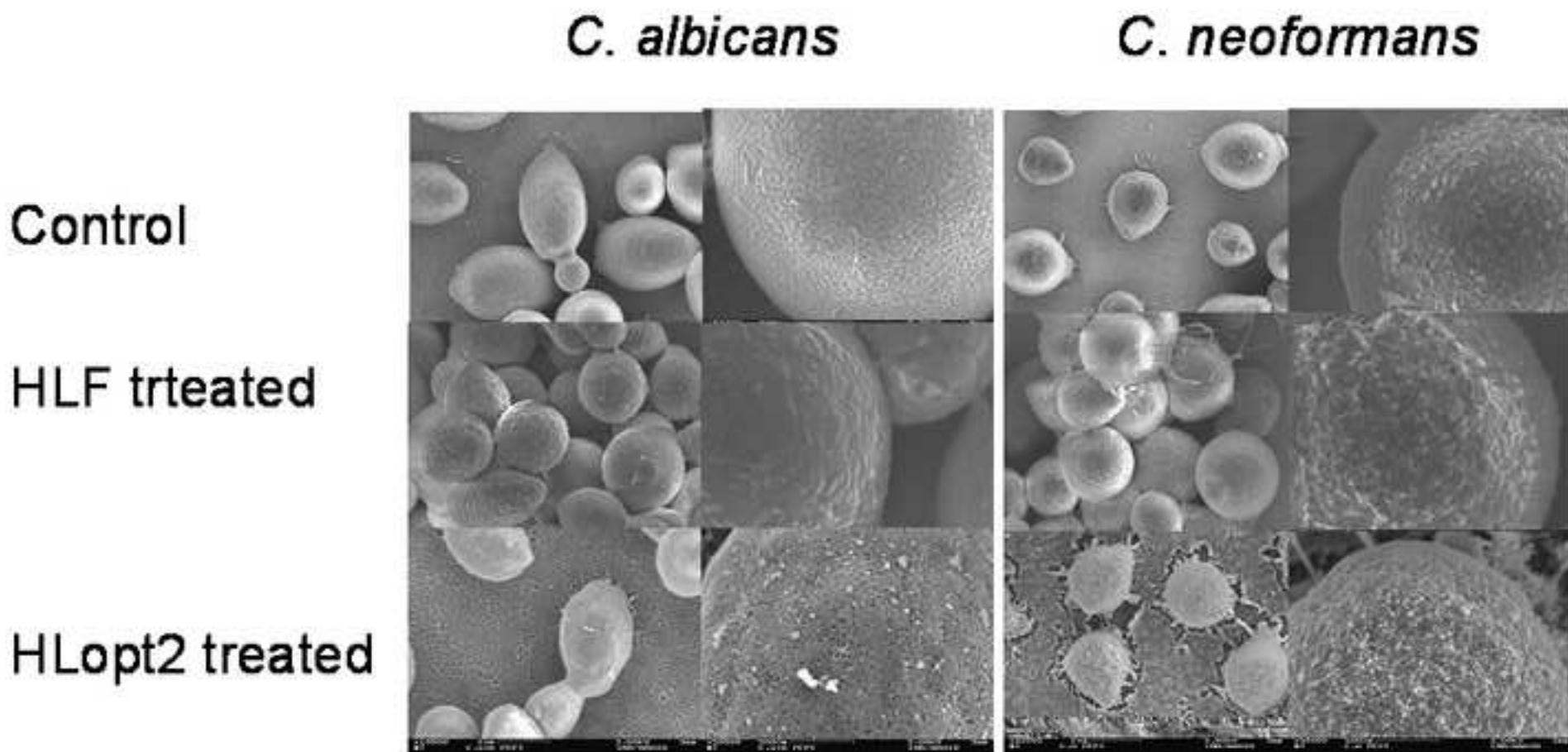


Fig 4

