Antimicrobial activity of cathelicidin peptides and defensin against oral yeast and bacteria

JH Wong, TB Ng *, RCF Cheung, X Dan, YS Chan, M Hui

KEY MESSAGES

1. Human cathelicidin LL37 and its fragments LL13-37 and LL17-32 were equipotent in inhibiting growth of *Candida albicans*.
2. LL13-37 permeabilised the membrane of yeast and hyphal forms of *C albicans* and adversely affected mitochondria.
3. Reactive oxygen species was detectable in the yeast form after LL13-37 treatment but not in untreated cells suggesting that the increased membrane permeability caused by LL13-37 might also lead to uptake of the peptide, which might have some intracellular targets.
4. LL37 and its fragments also showed antifungal activity against *C krusei*, and *C tropicalis*.
5. A 5447-Da antifungal peptide with sequence homology to plant defensins was purified from king pole beans by chromatography on Q-Sepharose and FPLC-gel filtration on Superdex 75. It inhibited growth of fungi, including *Mycosphaerella arachidicola*, *Saccharomyces cerevisiae* and *C albicans* with an IC_{50} value of 3.9, 4.0, and 8.4 μM, respectively. The peptide increased fungal membrane permeability.
6. LL37 did not show obvious antibacterial activity below a concentration of 64 μM and its fragments did not show antibacterial activity below a concentration of 128 μM. Pole bean defensin exerted antibacterial activity on some bacterial species.

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[1] JH Wong, [1] TB Ng, [1] RCF Cheung, [1] X Dan, [1] YS Chan, [2] M Hui

The Chinese University of Hong Kong:
[1] *School of Biomedical Sciences*
[2] *Department of Microbiology*

* Principal applicant and corresponding author:
b021770@mailserv.cuhk.edu.hk

Introduction

Fungal resistance to traditional drugs calls for new antimicrobial agents. In mammals, defensins and cathelicidins are two major families of antimicrobial peptides. Cathelicidins are cationic peptides with a conserved N-terminal cathelin-like domain and a variable C-terminal antimicrobial domain. Cathelicidins rapidly kill *Candida* and other yeast spp.[1] *Candida* infection with *C albicans* is the fourth most common cause of hospital-acquired bloodstream infection in the USA. Defensins are cationic cysteine-rich peptides found in mammals, fungi, insects, and plants.[2] Defensins interact with membrane phospholipids leading to compromised membrane permeability. Defensins gain entry into the cells to interact with intracellular targets and interfere with cellular activity.[3] This study aimed to (1) determine whether human cathelicidin LL37 and its fragments LL13-37 and LL17-32 can inhibit *C albicans*, and (2) purify an antifungal defensin from *Phaseolus vulgaris* cv. 'king pole bean' seeds to investigate its antimicrobial activity on the growth of pathogenic fungi and yeasts.

Methods

This study was conducted from November 2009 to January 2011. All peptides were synthesised using the solid-phase method of Fmoc chemistry. Cathelicidin and fragments were assayed for a cytotoxic effect on human peripheral blood monocytes and haemolytic effect on rabbit erythrocytes. Antifungal activity of LL37 and its fragments on *C albicans* and other *Candida* species was monitored using a XTT reduction assay.

Defensin was isolated from king pole beans by ammonium sulfate precipitation, chromatography on Q-Sepharose and gel filtration on Superdex 75. Molecular mass was determined by SDS-PAGE and mass spectrometry. Purified peptide was submitted to sequencing.

C albicans strains (SC5134 and ATCC 90028) were used. Yeast cells were cultured for 36 hours with or without LL13-37 before observation under a scanning electron microscopy (SEM).

To assay for permeabilisation of the fungal membrane, *C albicans* cultures were incubated with 5 μM LL13-37 at 37°C for 8 hours. SYTOX Green was then added up to 0.5 μM. After 10 minutes, cells were observed under a confocal microscope.[4] SYTOX Green, a high-affinity nuclear stain, penetrates cells with a compromised membrane.

To localise mitochondria in *C albicans* cells, the cells were cultured for 12 hours in RPMI 1640

with or without LL13-37, and washed with PBS. Mitotracker deep-red was added and incubated for 30 minutes. The medium was aspirated and cells were washed twice. Images were acquired using a confocal microscope.

C albicans cells were treated with bimane-labelled cathelicidin peptides and visualised under a confocal microscope (excitation: 330-385 nm).[5]

To measure reactive oxygen species production, *C albicans* cells were cultured for 24 hours with or without LL13-37, and washed. After incubation with 25 μM5-(and-6)-carboxy-2',7'-dihydrodichlorofluorescein diacetate (carboxy-H_2DCFDA) in PBS at 37°C for 30 minutes, the cells were washed and imaged using a confocal microscope.[6]

To assay induction of cytokine gene expression, cells were isolated from the spleen of BALB/c mice (20-25 g) by pressing the tissue through a sterilised stainless steel sieve and were resuspended in culture medium.[1] A 4-mL aliquot of cells was incubated with 10 mL medium containing 0.1 mL 2 μM isolated peptide solution and Con A, at 37°C in 5% CO_2 for 4 hours. Total RNA extraction was performed using TRIzol method followed by RT-PCR. Agarose gel electrophoresis was performed to visualise the PCR product.

To assay antibacterial activity, sterile Petri plates containing 10 mL of nutrient agar (8% agar) were used. A 3-mL aliquot of warm nutrient agar (0.7%) containing the bacterium was poured onto the plates. A sterile blank paper disk (0.625 cm in diameter) was placed on the agar. The test sample (10 μL) was added to the disk. The plate was incubated at 37°C for 24 hours. A transparent ring around the paper disk signified antibacterial activity.

Antibacterial activity against *Pseudomonas aeruginosa*, *Streptococcus sanguis*, *Staphylococcus aureus*, *Porphyromonas gingivalis*, and *Haemophilus influenzae* was assessed via broth macrodilution using Clinical and Laboratory Standards Institute methodology. Stock solutions of peptides were prepared to a concentration of 500 μg/mL in buffer. Organisms were subcultured and isolated on blood agar, suspended in 3 mL of Mueller-Hinton broth at a turbidity of a 0.5 M McFarland standard, and diluted to approximately 10^5 CFU/mL before introduction into a 96-well plate containing serially two-fold diluted peptides in Mueller-Hinton broth. The turbidity resulting from peptides solution in broth required the creation of a control well lacking microbes to serve as turbidity control. The plate was incubated overnight for 16-20 hours at 37°C.

Results

Characteristics of human cathelicidin and fragments

Treatment of human peripheral blood monocytes with 125 μM to 1 μM LL 37, LL13-37, and LL17-32 resulted in viability of 83-92%. There was no haemolytic effect on rabbit erythrocytes at 1 to 25 μM (91-99% viability), but LL37 and LL17-32 exerted a haemolytic effect at 125 μM.

Following exposure of cells to LL13-37, LL37, and LL17-32 for 24 hours at 37°C on a 96-well polystyrene plate, XTT assay revealed that *C albicans* cells were inhibited. The ranking of potencies was LL37 > LL13-37 > LL17-32.

On SEM images of *C albicans* (Fig 1), LL13-37, LL37, and LL13-37 had analogous potencies in inhibiting hyphal growth in *C albicans*. The hyphae were uniformly thick in the negative control. After treatment with LL13-37, the hyphae assumed a more slender appearance. Budding appeared less robust and death ensued. Following exposure to 50 μM LL 13-37, only the yeast form and no hyphal form was discernible (Fig 1).

LL13-37 at 5 μM permeabilised the membrane of the yeast and hyphal forms since the nuclear stain SYTOX Green was localised in both forms. There was no green fluorescence in the PBS-treated yeast or hyphal form.

Mycelia incubated with LL13-37 were stained by SYTOX green, but not by MitoTracker deep red, indicating mitochondria were not adversely affected by LL13-37 (Fig 2).

Bimane-labelled LL13-37 entered some but

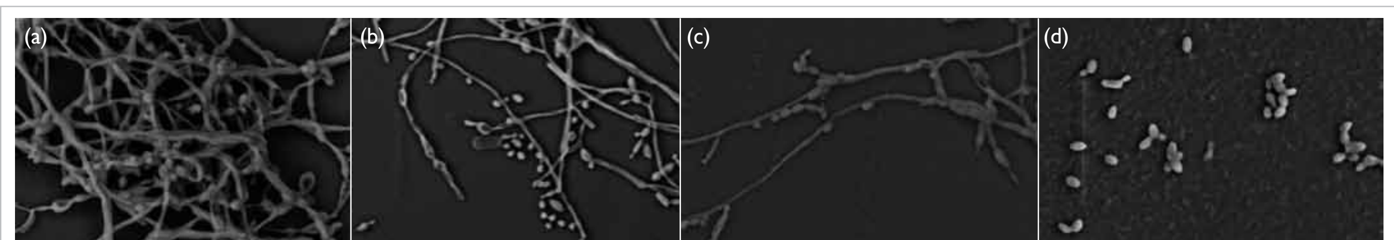

FIG 1. Scanning electron microscopic images of *C albicans* showing hypha formation in different LL13-37concentrations: LL 37 and LL13-37 have similar growth-inhibiting potencies (not shown). (a) In negative control (0 μM), hyphae were uniformly thick. In (b) 0.5 μM and (c) 5 μM LL13-37, hyphae became slender, budding became less healthy-looking and death resulted. (d) In 50 μM LL 13-37, only planktonic form and no hyphal form was discernible

not all of the *C albicans* hyphae.

Reactive oxygen species were demonstrable by confocal microscopy in the yeast form of *C albicans* cells after treatment with LL13-37, but not in the untreated cells. The difference due to LL13-37 treatment of the hyphal form was much less conspicuous and is not presented.

Respectively for *C albicans* (SC5134), *C albicans* (ATCC 90028), *C krusei* (ATCC 6258), and *C tropicalis* (ATCC 750), IC_{50} values of LL37 were 0.25±0.02, 0.56±0.08, 1.29±0.05, and 0.64±0.02 μM, whereas IC_{50} values of LL13-37 were 0.51±0.01, 0.67±0.06, 1.89±0.03, and 1.21±0.12 μM, and IC_{50} values of LL17-32 were 1.02±0.02, 1.51±0.12, 3.22±0.11, and 2.51±0.08 μM. LL37 did not show obvious antibacterial activity below a concentration of 64 μM and its fragments did not show antibacterial activity below a concentration of 128 μM.

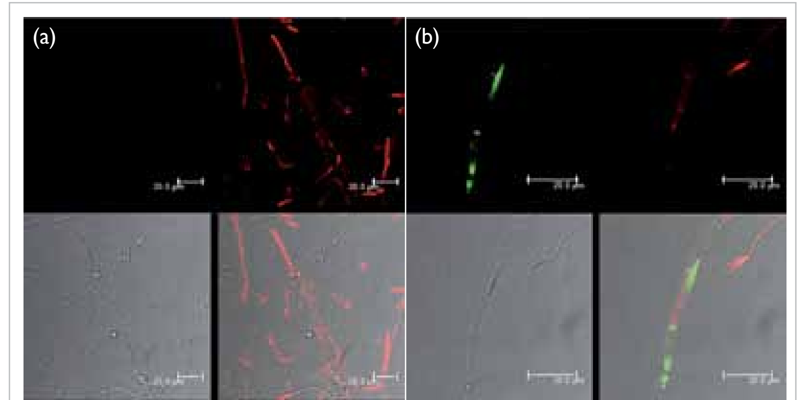

FIG 2. **Confocal microscope pictures showing staining of *C albicans* mycelia with SYTOX green and MitoTracker deep red: (a) control (treated with PBS), and (b) treated with 5 μM LL13-37.** Mycelia treated with LL13-37 stained with SYTOX green, but not with MitoTracker deep red. The upper left, upper right, lower left, and lower right quadrants refer to staining with SYTOX green, staining with MitoTracker deep red, bright field, and superimposed picture, respectively

Isolation and characterisation of bean defensin

Two peaks were demonstrated by Q-Sepharose chromatography. Anti-fungal activity in king pole bean seeds was recovered in the non-absorbed fraction, which was subsequently purified by gel filtration on Superdex 75. Three peaks were collected. Purified peptide (5 mg/50 g seeds) was located in fraction S3.

Molecular weight was determined by gel filtration on Superdex 75. The calibration curve was constructed using protein markers. Tricine gel electrophoresis of the peptide showed a single 5-kDa band (data not shown). Its accurate molecular mass was determined by mass spectrometry to be 5447.4 Da.

The purified peptide remained stable after exposure for 30 minutes from 0-80°C. However, its activity was lost after incubation for 30 minutes at or above 90°C. All activity remained at pH 4-10.

The N-terminal sequence of the antifungal peptide was KTCENLADTFRGPCFATSNC. It shared 75-89% identity with plant defensins.

The isolated peptide was active against *M arachidicola* and *S cerevisiae* with an IC_{50} value of 3.9 and 4.0 μM, respectively.

When the culture medium contained 5 mM $MgCl_2$, the IC_{50} value of the peptide to *M arachidicola* and *S cerevisiae* increased to 12 and 10.8 μM respectively.

Fluorescent microscopy revealed that the isolated peptide increased the membrane permeability in *S cerevisiae*, *M arachidicola*, and *C albicans* (Fig 3).

Only cytokine TNF-α was induced in murine splenocytes by the isolated peptide.

IC_{50} values of antibacterial activity of pole bean defensin toward *Mycobacterium phlei*, *Bacillus megaterium*, *Bacillus subtilis*, and *Proteus vulgaris* were 90±3, 100±7, 102±6, and 92±5 μM, respectively. For *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*, no inhibition was observed at a dose of 150 μM pole bean defensin.

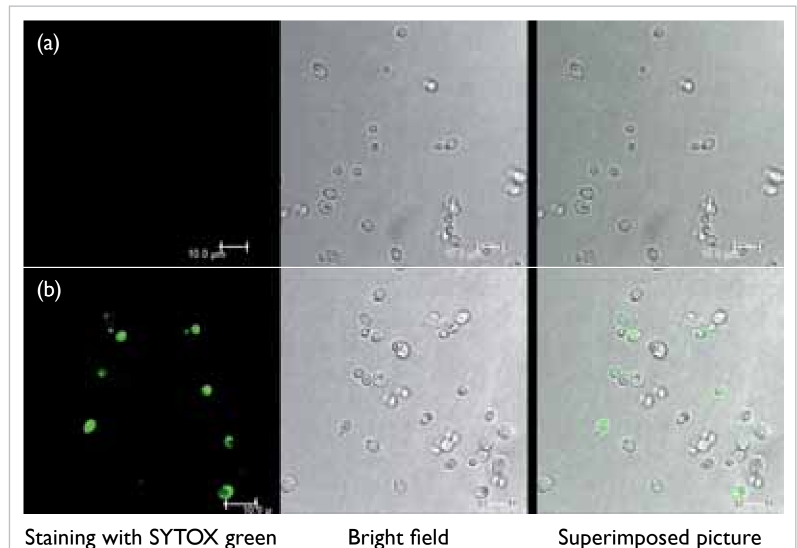

FIG 3. **Membrane permeabilisation assay of different forms of *C albicans* cells treated with 20 μM pole bean antifungal peptide: (a) treated with PBS as controls, (b) treated with peptide.** All fungal cells showed strong SYTOX green fluorescence in the presence of peptide, as compared with controls, in which fungi were grown without the peptide. All pictures were taken with a confocal microscope.

Discussion

LL13-37 was selected because of its lowest toxicity to human peripheral blood monocytes and rabbit

erythrocytes. Antimicrobial activity was most likely attributed to the cationic nature of LL37 and its fragments (LL13-37 and LL17-32) that carry +6, +4 and +5 net charges, respectively. Hence, they could interact with negatively charged components of the microbial surface and subsequently disrupt the membrane barrier via pore-formation or non-specific membrane permeabilisation. SYTOX Green, a high-affinity nucleic acid stain that does not traverse live cell membranes but penetrates cells with a compromised plasma membrane, is an indicator of membrane integrity. LL37 and fragments are positively charged at a neutral pH, α-helical, and contain many hydrophobic and basic residues, enabling them to bind and disrupt the negatively charged membrane of pathogens, culminating in cell death.

Findings on bimane-labelled LL13-37 revealed that LL13-37 enters fungal cells. The increased membrane permeability might not be the sole determinant of cell death but it might ensue in uptake of the peptides. Once inside cells, they may interfere with nucleic acid and/or protein synthesis. The peptides might have some intracellular targets.

Intracellular localisation of LL13-37 and its activity in *C albicans* cells was accompanied by reactive oxygen species accumulation, which can produce deleterious effects on nucleic acids, proteins and lipids. With this multiplicity of targets it is not easy to pinpoint the events that contribute to loss of cell viability following reactive oxygen species–induced damage.

After treatment with LL13-37, the mitochondria in *Candida* could not be labelled by Mitotracker deep red, a cell-permeant mitochondrion-selective dye. The results indicated that LL13-37 could enter *Candida* cells and adversely affect the mitochondrial membrane.

The isolated king pole bean defensin exerted potent antifungal activity against *S cerevisiae* and *C albicans* with an IC_{50} value of 4.0 and 8.4 μM, respectively. Its efficacy against *C albicans*, which can cause infection in immunocompromised patients, is noteworthy. The possibility of exploiting plant defensins as an antifungal therapy in humans has been proposed.[7] Its antifungal potency is similar to that reported for other plant defensins.

In the present study, fungi treated with the antifungal peptide were stained with SYTOX Green, a high-affinity nucleic acid stain that does not cross live cell membranes. Only cytokine TNF-α was induced in murine splenocytes by the isolated peptide suggesting that the peptide is a weak cytokine gene inducer. King pole bean defensin did not induce haemolysis in rabbit erythrocytes, indicating that it adversely affects fungal but not mammalian cell membranes. This enhances the therapeutic potential of the peptide.

Acknowledgements

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