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Antifungal activity of C3a and C3a-derived peptides against *Candida*

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Abstract

Antimicrobial peptides are generated during activation of the complement system [Nordahl et al. Proc. Natl. Acad. Sci. U. S. A. 2004, 101:16879-16884]. Here we show that the anaphylatoxin C3a exerts antimicrobial effects against the yeast *Candida*. Fluorescence microscopy and electron microscopy analysis demonstrated that C3a-derived peptides bound to the cell surface of *Candida*, and induced membrane perturbations and release of extracellular material. Various *Candida* isolates were found to induce complement degradation, leading to generation of C3a. Arginine residues were found to be critical for the antifungal and membrane breaking activity of a C3a-derived antimicrobial peptide, CNY21 (C3a; Cys^{57}–Arg^{77}). A CNY21 variant with increased positive net charge displayed enhanced antifungal activity. Thus, C3a-derived peptides can be utilized as templates in the development of peptide-based antifungal therapies.

Keywords: *Candida*; Atopic dermatitis; Innate immunity; Antimicrobial peptides; Complement; Structure–activity relationship study

1. Introduction

The innate immune system, based on antimicrobial peptides (AMP), provides a rapid and non-specific response against potentially invasive pathogenic microorganisms. At present, over 880 different AMPs have been identified in eukaryotes (www.bbcm.univ.trieste.it/~tossi/pag5.htm). The endogeneous AMPs cathelicidin LL-37/hCAP18, β-defensins, RNase 7, various dermcidins as well as psoriasin are found in skin [1–5]. The significance of cathelicidins for bacterial clearance is exemplified by findings indicating that the mouse antibacterial cathelicidin peptide CRAMP protects the skin from invasive bacterial infection [6]. Furthermore, molecules previously not considered as AMPs, including proinflammatory and chemotactic chemokines exert antibacterial and antifungal activities [7]. The proinflammatory, chemotactic, and anaphylatoxic peptide C3a, generated during activation of the complement system [8,9] displays potent antibacterial effects [10]. In general, AMPs interact with microbial membranes and subsequent events involve membrane destabilization and permeabilization and/or interaction with intracellular targets, ultimately leading to microbial killing [11–16]. Structural prerequisites for peptide action have been studied in great detail in several structure–activity relationship studies, and multiple parameters including conformation, net charge, size, degree of amphipathicity and hydrophobicity govern the antimicrobial activity of AMPs [11,17–19]. Structural differences between bacterial, fungal, and mammalian cell surfaces underlie a certain degree of selectivity for AMP action and many peptides may preferentially target

Abbreviations: AMP, antimicrobial peptides; CF, carboxyfluorescein; cfu, colony-forming units; DOPA, 1,2-Dioleoyl-*sn*-Glycero-3-Phosphate, monosodium salt; DOPC, 1,2-dioleoyl-*sn*-Glycero-3-phosphocholine; Low-EEO, low-electroendosmotisistype agarose gel; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MEC, minimal effective concentration; RDA, radial diffusion assay; TAMRA, Tetramethylrhodamine; YPD, yeast extract-peptone-dextrose

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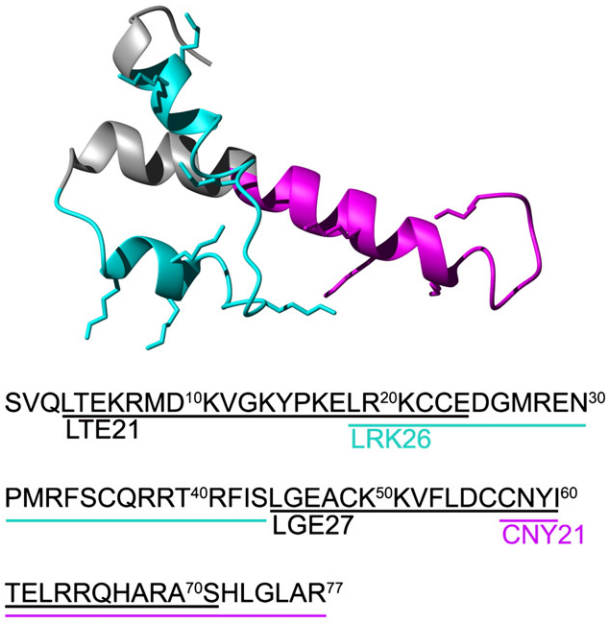

Fig. 1. Sequence and molecular model of C3a and peptides used in the study. The peptides LTE21, LRK26, LGE27 and CNY21 are indicated in the sequence. The peptides corresponding to regions CNY21 (violet) and LRK26 (blue) exerted anticandidial activity. Lysine and arginine residues of these regions are visualized.

bacteria, fungi, or mammalian cells or combinations thereof [16,20]. Thus, bacterial surfaces contain many anionic components, including LPS and anionic lipids of Gram-negative bacteria, as well as teichoic and teichuronic acids of Gram-positive bacteria. Similarly, β-glucan, chitin, manno-protein and a blend of other cell wall proteins and polysaccharides contribute to a negative surface potential of fungal surfaces [21–23]. Beyond the outer cell surface, AMPs interact with the plasma membrane. Contrasting to eukaryotic membranes, including fungal membranes, which contain mostly zwitterionic lipids (such as phosphatidylcholine), bacterial membranes comprise various acidic phospholipids (phosphatidylglycerol, phosphatidylserine and cardiolipin), which confer a negative charge facilitating AMP binding and sometimes also defect formation [5,12,16]. The plasma membrane of eukaryotic cells contains sphingolipids and sterols, which are missing in prokaryotes [24], and which are frequently found to provide some resistance to AMP membrane rupture [5,25,26]. Ergosterol is the major sterol in yeasts, whereas the sterol in plasma membranes of mammalian cells is represented by cholesterol [24]. All these factors contribute to AMPs exhibiting different activity spectra on bacterial and fungal membranes, as well as toxicity on eukaryotic cells. It has, however, become increasingly clear that AMP selectivity may also depend on factors such as AMP oligomerisation and preassembly (in solution and membrane) [27].

Candida is a dimorphous fungus colonizing the mucous membranes of the mouth and vagina in a saprophytic manner. However, *Candida* is also known to be involved in several diseases such as cutaneous infections, atopic eczema, oroesophageal candidiasis, candida vaginosis and septicaemia [21,28]. In animal models of cutaneous candidiasis it has been shown that *Candida* activates the complement system and C3 fragments are deposited at the cell surface of Candida [29–31]. In humans, generation of C3a has been implicated in the pathogenesis of skin diseases, such as atopic dermatitis [32,33]. Conversely, *C. albicans* may also inhibit the alternative and classical pathways of complement activation by binding factor H, FHL-1, and C4b-binding protein [34,35].

In the present study we investigated whether C3a-like peptides, generated in response to *Candida* infection, exert antifungal effects. Our data disclose a novel antifungal activity of C3a and functional epitopes of C3a. Amino acid alterations in a C3a-derived peptide were performed to elucidate structural requirements for peptide action as well as enhance peptide activity. The findings can be utilized in the further development of C3a-derived peptides for therapeutic use.

2. Materials and methods

2.1. C3-derived peptides and LL-37

C3a was obtained from Calbiochem. The peptides LTE21, LRK26, LGE27, CNY21, CNY21H-K, CNY21H-L, CNY21H-P, CNY21R-S, LL37, (Fig. 1 and Table 1), Tetramethylrhodamine (TAMRA)-conjugated CNY21 and LRK26 were all synthesized by Innovagen AB (Lund, Sweden). The purity (>95%) and molecular weight of these peptides was confirmed by mass

Table 1
Complement factor C3-derived peptides analysed in the study

Peptide	Sequence	Mw (Da)	pI [a]	Net charge	AGADIR [b]	μHrel [c]
CNY21	CNYITELRRQHARASHLGLAR	2465.83	10.7	+3	4.8	0.28
CNY21H-K	CNYITELRRQ**K**ARAS**K**LGLAR	2447.89	10.9	+5	9.7	0.27
CNY21H-L	CNYITELRRQ**L**ARAS**L**LGLAR	2417.87	10.7	+3	17.9	0.34
CNY21H-P	CNYITELRRQ**P**ARAS**P**LGLAR	2385.78	10.7	+3	1.9	0.28
CNY21R-S	CNYITEL**SS**QHA**S**ASHLGLAR	2258.5	6.9	0	0.6	0.24

Mw, molecular weight. Da, Dalton. The parameters are set at pH 7.40, temperature 278 K (5 °C), ionic strength 0.150 M, N-terminal and C-terminal free. Bold letters indicate amino acid substitutions.

[a] pI, the theoretical isoelectric point calculated by using the Protparam tool available at http://us.expasy.org/tools/protparam.html.

[b] Agadir is a prediction algorithm based on the helix/coil transition theory that predicts the helical behavior of monomeric peptides available at http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html.

[c] Relative hydrophobic moment (μHrel) was calculated by using HydroMCalc (Kyte and Doolittle) available at http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html.

spectral analysis (MALDI-TOF Voyager, Applied Biosystems, Foster City, CA, USA).

2.2. Assays for candidacidal activities

C. albicans ATCC 90028 was obtained from the Department of Bacteriology at Lund University Hospital. Clinical *Candida albicans* and *parapsilosis* isolates were obtained from patients with atopic dermatitis. Cells were precultured over night on Sabouraud–dextrose–agar (Difco, Detroit, MI) at 28 °C, inoculated in 10 ml yeast extract–peptone–dextrose (YPD) broth (Sigma-Aldrich, St. Louis, USA) at 28 °C and grown to mid-logarithmic phase. The cells were washed three times and diluted in 10 mM Tris, pH 7.4. Fifty μl of a suspension containing 1×10^6 fungal cfu/ml was incubated at 28 °C for 2 h with C3a, LL37, or the peptides CNY21, CNY21H-K, CNY21H-L at concentrations ranging from 0 to 6 μM (for C3a) and 0 to 30 μM (for the other peptides). To quantify the fungicidal activity, serial dilutions of the incubation mixture were plated onto Sabouraud-dextrose-agar (Difco, Detroit, MI) plates and incubated 48 h at 28 °C, the number of colony-forming units (cfu) were thereafter determined by counting visible colonies.

Radial diffusion assay (RDA) was performed as described by Lehrer et al. [36] with some minor modifications. *Candida* was grown for 16 h at 28 °C in 10 ml YPD to obtain yeast form organisms. The culture was then centrifuged at $2000\times g$ for 10 min, washed three times with 10 mM Tris, pH 7.4. A volume containing 1×10^5 cfu was added to 5 ml of previously autoclaved, warm 42 °C, underlay agarose gel that contained 1.5 mg of TSB, 50 mg of

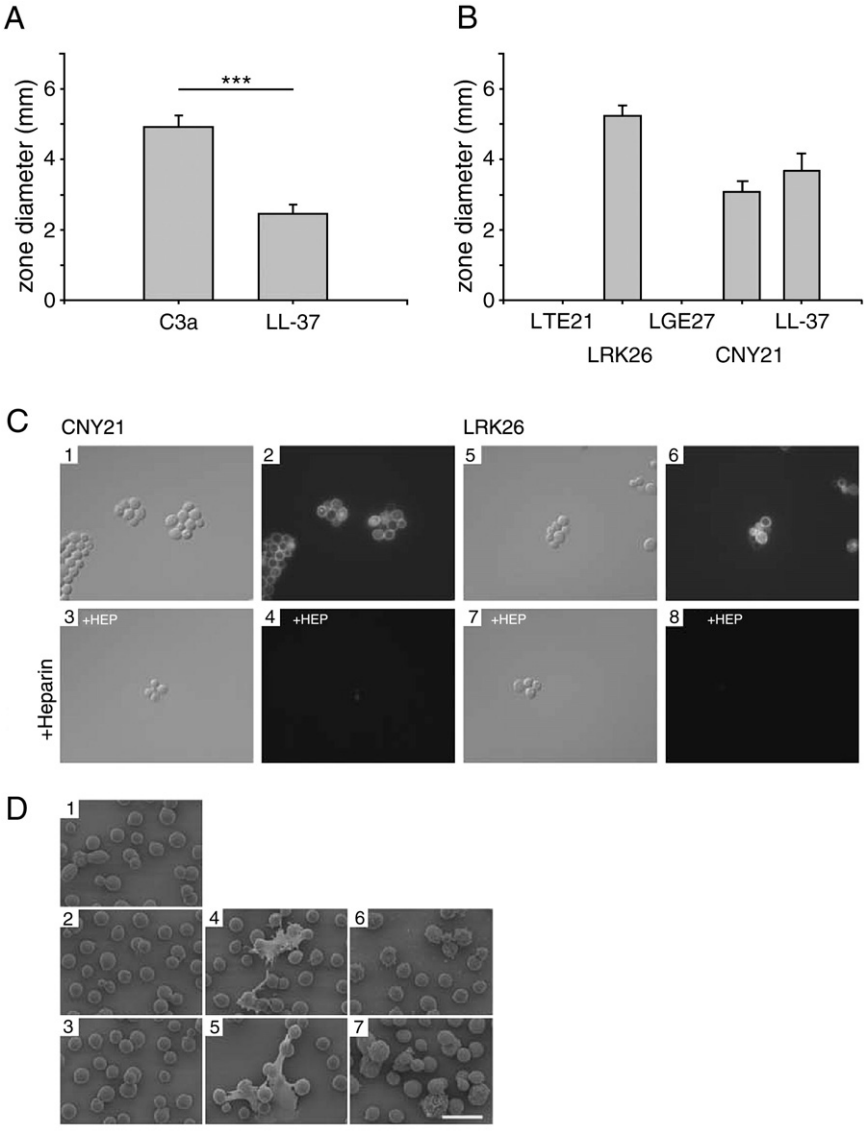

Fig. 2. Antimicrobial activity of C3a and C3a-derived peptides against *C. albicans* ATCC 90028. (A) C3a and LL37 were tested in RDA, each well was loaded with 6 μl with 50 μM of the peptides. Standard error of the mean (SEM) are indicated. C3a was significantly more potent than the control peptide LL37; ***indicates a statistically significant difference ($P<0.001$; $n=9$). (B) The indicated peptides (6 μl each at 100 μM), spanning different helical regions of the C3a molecule, were tested in RDA using *C. albicans*, LRK26 and CNY21 showed anticandidial activity. For comparison LL37 was used ($n=8$). (C) Binding of TAMRA-CNY21 and TAMRA-LRK26 to *Candida albicans* ATCC 90028 and inhibition of binding by heparin. Panels 2 and 6 shows red fluorescence of *Candida* cells stained with TAMRA-conjugated peptides (10 μg ml^{-1}) and panels 4 and 8 show yeast cells incubated with heparin and the corresponding TAMRA-conjugated peptides. Images in panels 2, 4, 6, and 8 were recorded using identical instrument settings. The corresponding Nomarski images are shown in panels 1, 3, 5 and 7. (D) Treatment of *Candida* with C3a-derived peptides and LL-37. *Candida* cell suspensions (1×10^6) were incubated with the peptides, absorbed to glass coverslips and visualized by scanning electron microscopy. LL37 and CNY21R-S served as positive and negative controls, respectively. Panel 1 shows *Candida* cells alone, Panels 2, 4, and 6 show effects of 50 μM of CNY21R-S, CNY21 and LL-37, respectively and panels 3, 5, and 7 show the corresponding peptides at 100 μM. Scale bar represents 10 μm.

low-electroendosmosistype (Low-EEO) agarose gel (Sigma-Aldrich Inc, St. Louis, MO USA, A-6013-25G) and 1 μl of Tween 20 (0.02%w/v) (Sigma Chemical Co.) dissolved in 10 mM Tris, pH 7.4. The fungi were directly dispensed into the agar and mixed with a vortex mixer for 10 s and then quickly poured out into a Petri dish, 85 mm in diameter. Wells, 4 mm in diameter, were punched and each well was then filled up with 6 μl of test sample. After incubation in 35–37 °C for 3 h, 5 ml of overlay agarose gel, consisting of 300 mg TSB, 50 mg Low-EEO dissolved in distilled sterilized water, was poured out on top of the underlay agarose gel. A clear zone surrounding the wells indicated antibacterial activity and was measured after 18–24 h of incubation at 28 °C. The minimal effective concentration (MEC) was obtained by plotting the zone diameter and logarithmic concentration of the peptides, then using the *x* intercept value calculated from a linear regression analysis in SigmaStat (Systat Software Inc., Point Richmond, CA, USA).

2.3. Fluorescence microscopy

C. albicans ATCC 90028 was incubated for 17 h in 10 ml YPD-broth at 28 °C to obtain mid-logarithmic phase organisms and washed three times, then diluted in 10 mM Tris, pH 7.4 to a concentration of 1×10^7 cfu/ml. Two hundred μl samples of the *C. albicans* suspension was incubated for 5 min on ice together with 1 μl TAMRA-CNY21 or TAMRA-LRK26 (2 mg/ml). For blocking of binding of CNY21 and LRK26 to the fungal membrane, 2 μl heparin (50 mg/ml) was added to the samples prior to incubation with TAMRA-CNY21 and TAMRA-LRK26. The pellets were washed three times in 10 mM Tris, pH 7.4. Cell FIX (Becton Dickinson Catalogue No. 340181) was added (to a final concentration of 4% formaldehyde) and the yeast cell pellets were incubated 15 min on ice and then 45 min in room temperature. Ethanol washed glass cover slips were coated with 0.25 ml of poly-lysine (0.2 mg/ml dissolved in water), dried and then washed with distilled water. The samples were adhered to the poly-lysine glasses for 30 min and mounted on microscope slides for visual inspection. A Nikon Eclipse TE300 inverted fluorescence microscope was used.

2.4. Electron microscopy

C. albicans ATCC 90028 cells (1×10^6 cells per sample) were incubated for 2 h with 50 μM and 100 μM of CNY21R-S, CNY21 or LL-37. *C. albicans* cells incubated in 10 mM Tris buffer, pH 7.4 only, were included for control. The samples were fixed in 2.5% (v/v) glutaraldehyde in 0.15 M sodium cacodylate, pH 7.2, incubated at room temperature over night and washed 4×10 min in 0.15 M sodium cacodylate, pH 7.2. Samples were washed with cacodylate buffer, and dehydrated with an ascending ethanol series from 50% (v/v) to absolute ethanol (10 min per step). The material was then critical point dried in carbon dioxide, with absolute ethanol as intermediate solvent, mounted on aluminium holders and sputtered with 50 nm palladium/gold. They were examined in a JEOL JSM-350 scanning electron microscope operated at 5 kV.

2.5. Liposome preparation and leakage assay

Dry lipid films were prepared by dissolving either dioleoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) (60 mol%) and cholesterol (Sigma, St. Louis, MO) (40 mol%; added for reducing spontaneous liposome leakage to less than a couple of % over the time-scale of the experiment), or dioleoylphosphatidic acid (Avanti Polar Lipids, Alabaster, AL) (30 mol%), dioleoylphosphatidylcholine (30 mol%), and cholesterol (40 mol%) in chloroform, and then removing the solvent by evaporation under vacuum overnight. Subsequently, buffer (10 mM Tris, pH 7.4) was added together with 0.1 M carboxyfluorescein (CF) (Sigma, St. Louis, MO). After hydration, the lipid mixture was subjected to eight freeze–thaw cycles consisting of freezing in liquid nitrogen and heating to 60 °C. Unilamellar liposomes, of about ∅140 nm were generated by multiple extrusions through polycarbonate filters (pore size 100 nm) mounted in a LipoFast miniextruder (Avestin, Ottawa, Canada) at 22 °C. Untrapped carboxyfluorescein was then removed by two gel filtrations (Sephadex G-50) at 22 °C, with Tris buffer as eluent.

In the liposome leakage assay, self-quenching of CF was used. Thus, at 100 mM CF is self-quenched, and the recorded fluorescence intensity from liposomes with entrapped CF is low. On leakage from the liposomes, released CF is dequenched, and hence fluoresces. The CF release was determined by monitoring the emitted fluorescence at 520 nm from a liposome dispersion (10 mM lipid in 10 mM Tris pH 7.4). An absolute leakage scale is obtained by disrupting the liposomes at the end of the experiment through addition of 0.8 mM Triton X100 (Sigma, St. Louis, MO), thereby causing 100% release and dequenching of CF. A SPEX-fluorolog 1650 0.22-m double spectrometer (SPEX Industries, Edison, NJ) was used for the liposome leakage assay. Measurements were performed at 37 °C. The liposomes investigated were either zwitterionic (DOPC/cholesterol 60/40 mol/mol) or anionic (DOPC/DOPA/cholesterol 30/30/40 mol/mol). DOPA (1,2-Dioleoyl-*sn*-Glycero-3-Phosphate, monosodium salt) and DOPC (1,2-dioleoyl-*sn*-Glycero-3-phoshocholine) were both from Avanti Polar Lipids (Alabaster, USA) and of >99% purity, while cholesterol (>99% purity), was from Sigma-Aldrich (St. Louis, USA).

2.6. Generation of C3a-derived peptides in human serum

Human serum was incubated with *C. albicans* strain ATCC 90028 (1×10^8 cells) or clinical Candida isolates for up to 4 h at 28 °C in polypropylene tubes (total volume of 1 ml). For kinetic study of the effects of *C. albicans* strain ATCC 90028, 150 μl was drawn from the mixture at 0, 1.5, and 4 h and then centrifuged at 14,000 rpm. The pellet, containing *Candida* cells was washed three times in 10 mM Tris, pH 7.4 and stored in −20 °C. The supernatants were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 16.5% Tris–Tricine gel (Bio-Rad Laboratories, Hercules, CA) under reducing conditions, and then transferred onto nitrocellulose membranes (Hybond-C, Amersham Life Sciences). Membranes were blocked using 3% (w/v) skimmed milk (ICN Biomedicals Inc., Ohio, USA) in blocking buffer (50 ml 1 M Tris, pH 7.4, 10 ml Tween 20, 43.8 g NaCl in 500 ml water) and then washed in the buffer three times. The membranes were then incubated with rabbit polyclonal antibodies (Innovagen) against the peptide LGE27 (at 1:1000). After three washes the membranes were incubated with horseradish peroxidase-conjugated swine anti-rabbit secondary antibodies (DAKO A/S, Glostrup, Denmark) (1:1000). The proteins reacting with the antibodies were detected by using enhanced chemiluminescence ECL Western Blotting Detection Reagents Kit (Amersham Biosciences, Buckinghamshire, UK).

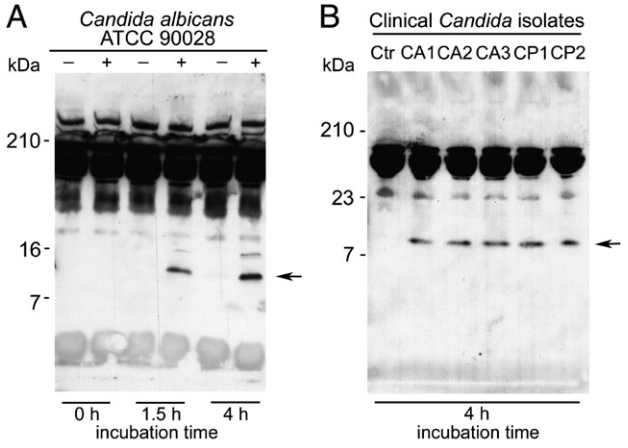

Fig. 3. Generation of C3a-derived peptides in human serum. Human serum was incubated with *Candida* ATCC90028, and C3a-peptides were detected by immunoblotting using polyclonal antibodies against the C3a-derived peptide LGE27. (A) Serum was incubated for the indicated time periods (bottom) in absence (−) or presence (+) of *Candida albicans* ATCC 90028. (B) Serum was incubated for 4 h alone (Ctr) or with various clinical isolates of *Candida albicans* (CA1–3) and *Candida parapsilosis* (CP 1 and 2) derived from the skin of patients with atopic dermatitis. In both panels, molecular mass markers are indicated on the left. The arrow indicates the position of intact C3a.

2.7. Statistical analysis

Statistical analysis of the difference between zone diameters of C3a and LL-37 in RDA (Fig. 1A) was performed using the Mann–Whitney Rank Sum Test. Comparison of antifungal activity between CNY21 and the various synthesized CNY21 variants (Fig. 4B) was performed using Kruskal–Wallis One Way Analysis of Variance on Ranks and pair ways multiple comparison procedures (Tukey Test). $P<0.05$ was considered as a significant difference. The statistical software used was SigmaStat (Systat Software Inc., Point Richmond, CA, USA).

3. Results and discussion

To investigate whether the anaphylatoxin peptide C3a possessed antifungal activity, we investigated the effects of purified C3a on a *Candida albicans* isolate. The results showed that C3a (for sequence see Fig. 1) was indeed active against *Candida* and notably, the peptide exerted a stronger inhibitory effect in the RDA assay when compared with the classical AMP LL-37 (both at 50 μM) under the low-salt conditions used (Fig. 2A). In viable count assays, the concentrations of C3a and LL37 required to kill 50% of the microorganisms were calculated to 5.0 μM and 11.5 μM, respectively. In order to determine which regions of C3a were responsible for the antifungal activity, overlapping fragments (Fig. 1), encompassing different helical regions of the C3a molecule were synthesized and tested for antifungal activities. Whereas the peptides LTE21 and LGE27 displayed no antimicrobial effects, the peptides LRK26 and CNY21 (Fig. 1) exerted potent antifungal activities in RDA against *C. albicans* ATCC 90028 (Fig. 2B). To investigate whether the peptides CNY21 and LRK26 bind to *Candida*, the peptides were labelled with the fluorescent dye TAMRA and incubated with the fungal cells. As demonstrated by fluorescence microscopy, both peptides bound to the surface of *Candida*, and the binding was completely blocked by heparin (Fig. 2C). Heparin did not quench the fluorescence of the TAMRA label (not shown). The well-defined carboxyterminal region of C3a, comprising CNY21 may adopt a helical conformation [9], and harbors typical features of helical AMPs [17]. Thus, in mice, a peptide corresponding to this region was protective against infection by the significant human pathogen *Streptococcus pyogenes* [10]. Furthermore, human neutrophilic enzymes release peptides exerting antimicrobial effects which

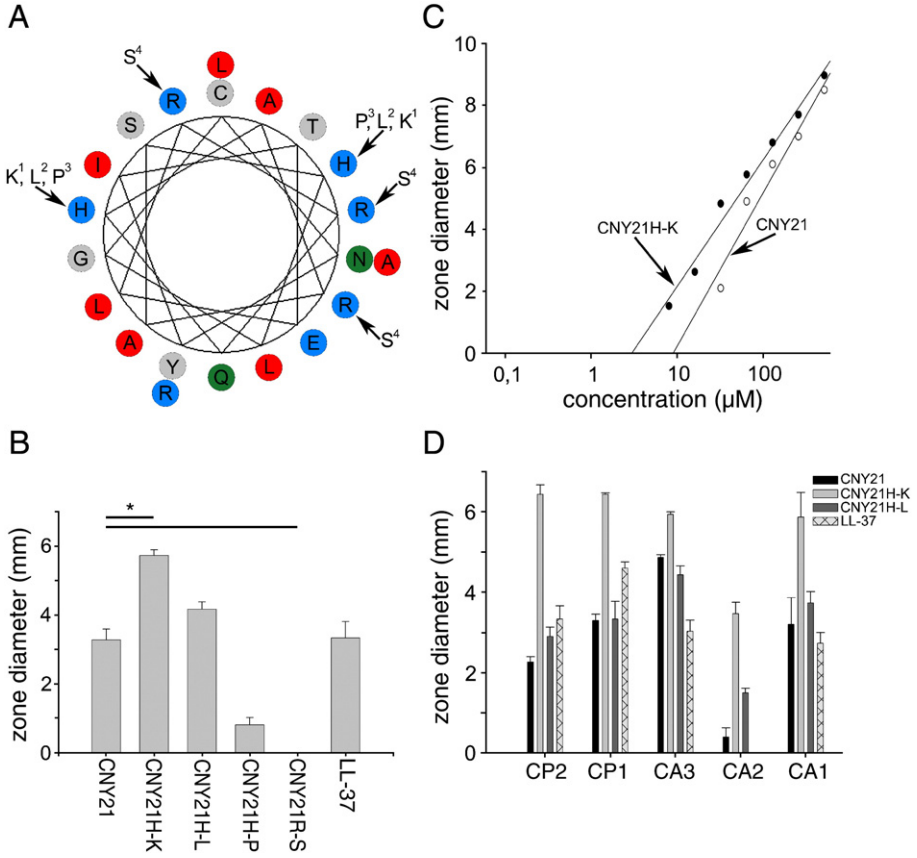

Fig. 4. Antifungal activities of CNY21 and variants thereof. (A) The helical wheel projection shows CNY21 assuming an α-helical conformation. The substituted amino acids in the modified variants [1]CNY21H-K, [2]CNY21H-L, [3]CNY21H-P and [4]CNY21R-S are indicated. Hydrophobic amino acids are represented by red and charged amino acids by blue circles. The uncharged Y, G, S, C, and T and the polar amino acids N and Q are indicated by grey and green circles, respectively. See also Table 1 for sequences. (B) RDA analysis of antifungal effects of the CNY21 variants. All peptides were tested at 100 μM. CNY21H-K yielded significantly larger inhibition zones than native CNY21, whereas CNY21R-S was inactive, * indicates a statistically significant difference ($P<0.05$; $n=11$). (C) The minimal effective concentrations (MEC) of CNY21 and CNY21H-K were investigated by RDA. The MEC-values are defined by the intersections of the regression lines with the *x* axis. MEC values obtained were 8.9 μM and 2.9 μM for CNY21 and CNY21H-K, respectively. Note that the scale of the *x* axis (concentrations of the peptides) is logarithmic. (D) Antimicrobial activity of CNY21 variants (100 μM) against clinical isolates of *Candida albicans* (CA 1–3) and *Candida parapsilosis* (CP 1 and 2). For all isolates, CNY21H-K yielded larger inhibition zones than CNY21 and LL-37. Error bars indicate SEM.

contain the carboxyterminal region of C3a (comprising CNY21), proving the physiological importance of this antimicrobial region of C3a [10]. Thus, the following experiments focused on CNY21. To further analyze the effects of this peptide on the yeast cells, *Candida* cells were incubated with the peptide CNY21, and analyzed by scanning electron microscopy. The human cathelicidin LL-37 was included for comparison and a CNY21-derived peptide containing the anaphylatoxin determinant LGLAR, but totally devoid of antibacterial activity [10], CNY21R-S (Table 1), was used as negative control. Compared with the control cells, CNY21-treated *Candida* cells displayed extracellular material and membrane perturbations. The cells treated with LL-37 showed significant perturbations of the cell surface, although less extracellular material was detected (Fig. 2D).

Having shown the antifungal activities of C3a and derived peptides, we explored whether *Candida* could generate similar C3a-like peptides in human serum. Western blot analysis using antibodies against the C3a-epitope LGE27 (containing a major part of CNY21), demonstrated that peptides corresponding to C3a were indeed formed in serum subjected to *Candida albicans* ATCC 90028 (Fig. 3A). A similar finding was obtained with five other clinical *Candida* isolates derived from patients with atopic dermatitis (Fig. 3B), thus presenting a proof of the concept that antimicrobial peptides may indeed be generated in response to *Candida* infection. Clearly, considering the multifunctionality of C3a, as well as the high redundancy of innate immune defences, the physiological *in vivo* significance of the herein presented direct antifungal activity of C3a needs to be determined. C3 is found in saliva as well as in vaginal fluid [37,38]. In blood, the concentration of C3 is 5–11 μM. Furthermore C3 is produced by endothelial cells, keratinocytes and monocytes [39–41]. Hence, complement activation in response to *Candida* infection should therefore induce generation of C3a at antifungal concentrations.

Various factors, such as net charge, hydrophobicity, and degree of amphipathicity govern the activity of AMPs. Concerning the group of peptides comprising α-helices, helix-stabilizing or destabilizing amino acids affect both antimicrobial as well as hemolytic activities [17,42]. Notably, introduction of polar or charged amino acid residues at hydrophobic regions of AMPs may result in improved therapeutic index, i.e. increase of antimicrobial activity with no concomitant increase of hemolysis [43]. In order to determine the structural requirements of CNY21 and its actions against *Candida*, we modified the net charge, hydrophobicity, as well as helical propensity of the peptide CNY21 (Table 1 and Fig. 4A). Replacement of the two histidine residues by lysine (CNY21H-K) significantly increased the antifungal activity, whereas the peptide CNY21H-L exerted similar antimicrobial effects as the native peptide. The MEC values for CNY21 and CNY21H-K, determined by RDA, were 8.9 μM and 2.9 μM, respectively (Fig. 4C). Analogous results were obtained in viable count assays. The concentrations of CNY21 and CNY21H-K required to kill 50% of the microorganisms were 9.25 μM and 5.9 μM, respectively. Introduction of helix breaking proline residues (CNY21H-P) significantly reduced the activity of the peptide, suggesting that a helical conformation is a prerequisite for action on *Candida* membranes. Replacement of the central three arginines by the polar residue serine completely abolished the antimicrobial activity of CNY21 (Fig. 4B). Corroborating findings were obtained using five clinical *Candida* isolates (Fig. 4D). In all cases, CNY21H-K yielded zones significantly larger than those obtained for CNY21, CNY21H-L, or LL-37. Notably, one *Candida* isolate (CA2) was completely resistant to LL-37. For zwitterionic and anionic liposomes, the membrane-disruptive potency for CNY21-variants increased with increasing net positive charge and mean hydrophobicity of the peptide, and was completely lost on elimination of all peptide positive charges (Fig. 5). Interestingly, the H->P substituted peptide retained some activity on negatively charged liposomes. In this context, it should be emphasized that the purpose of using zwitterionic and anionic liposomes was not to mimic fungal or eukaryotic cells or bacterial membranes, respectively, but merely to monitor the

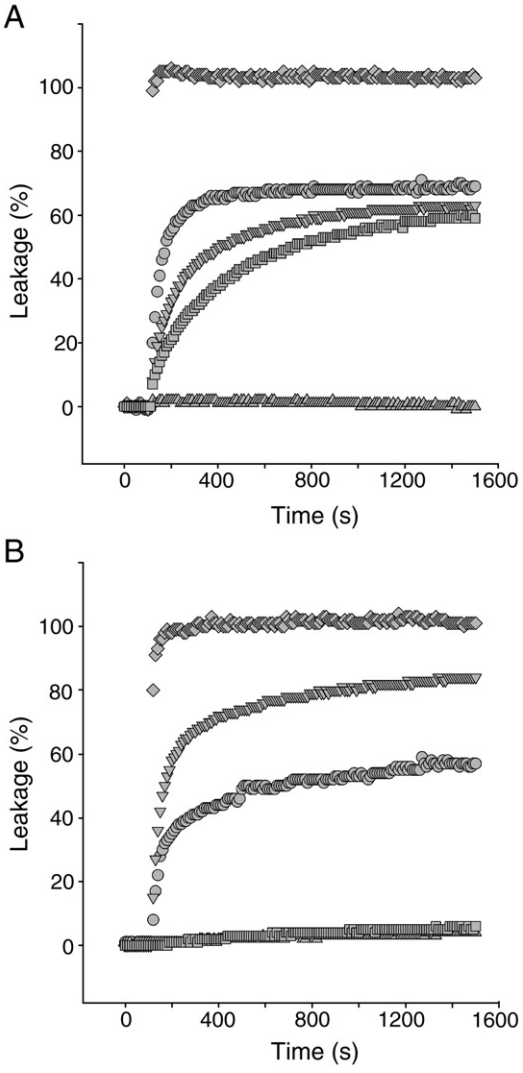

Fig. 5. CF leakage of from (A) anionic (DOPC/DOPA/cholesterol) and (B) zwitterionic (DOPC/cholesterol) liposomes induced by CNY21 (circle), CNY21H-L (diamond), CNY21H-K (triangle down), CNYH-P (square) and CNY21R-S (triangle up). In (B) the data sets of CNY21P and CNY21R-S overlap each other. The concentration of peptide used is 1 μM.

effects of electrostatics on the lipid–peptide interaction. Cholesterol was added to reduce spontaneous leakage from the liposomes to less than 2% during the time-scale of the experiment, in an effort to provide the highest possible quality of the leakage data. Nevertheless, the results are compatible with the current view of AMP functions, and indicate that a positive net charge, hydrophobicity, and a partial helical conformation are required for the antifungal and membrane-breaking action of CNY21.

Interestingly, neither CNY21 nor the CNY21 variants exerted any hemolytic effects on human erythrocytes and they did not permeabilize a human keratinocyte cell line [44]. The origin of the selectivity of the peptides investigated to fungi over other eukaryotic cells is unclear at present. Possibly, differences between erythrocytes and fungi in terms of sterol content could contribute to this increased peptide resistance of the former, as cholesterol possesses strongly membrane-stabilizing properties [45] and ergosterol has been found to induce less membrane stability in phospholipids than cholesterol [46]. Furthermore, although the z-potential of fungi and platelets is quite similar [47,48], this parameter depends sensitively on the material and charge distributions normal to the cell interface. It is therefore possible that differences in the latter may contribute to the observed selectivity as well, through differences in the local electrostatic peptide accumulation. Clearly, the exact mechanisms that determine the sensitivity of *Candida* to C3a-derived AMPs need further investigation. It is therefore interesting to note that Avrahami and Shai have demonstrated an approach for study of AMPs with antifungal activities using model membranes having a lipid composition resembling the bilayer of *C. albicans* [49,50]. Concludingly, the herein presented results indicate that C3a-derived peptides could be used in further studies of AMP interactions with *Candida* as well as provide interesting templates for the development of novel antifungal AMPs.

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