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Candidacidal activity of shortened synthetic analogs of amoebapores and NK-lysin

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Abstract Natural antimicrobial peptides and synthetic analogs thereof have emerged as compounds with potentially significant therapeutical application against human pathogens. Amoebapores are 77-residue peptides with cytolytic and antibacterial activity considered to act by forming ion channels in cytoplasmic membranes of the victim cells. A functionally and structurally similar peptide named NK-lysin exists in mammalian lymphocytes. Several synthetic analogs of amoebapores and NK-lysin, which are substantially reduced in size compared to the parent molecules, were tested for their ability to inhibit the growth of and to kill *Candida albicans*. Some of the peptides displayed potent activity against a clinical isolate as well as against defined culture strains. Among the most active peptides found are some shortened substitution analogs of amoebapore C and a cationic core region of NK-lysin. As these peptides are also highly active against Gram-positive and Gram-negative bacteria but are of low cytotoxicity towards a human keratinocyte cell line they may provide promising templates for the design of broad-spectrum peptide antibiotics.

Key words Amoebapore · Antimicrobial peptides · *Candida albicans* · NK-lysin · Synthetic peptides

Introduction

The emergence of microbial resistance to antibiotics has renewed the interest in natural antimicrobial agents. A widespread and crucial element of the first defense line of animals and plants against pathogens, and also a constituent of human innate immunity are antimicrobial peptides, many of which kill bacteria and fungi rapidly by permeating their membranes [1]. Chemical synthesis or recombinant expression permits the enhancement of particular properties of these natural bioactive molecules by changing their amino acid sequences; these techniques also allow the production of a given peptide in large quantities. Currently, the antimicrobial activity of many synthetic peptides are under investigation to employ them eventually against pathogens resistant to the classical antibiotics. The mode of action of these mostly cationic peptides, i.e., the physical disruption of microbial membranes, make them candidate molecules for new antibiotics since it has been assumed that their application will not create resistant strains [2]. A few synthetic antimicrobial peptides have already entered clinical trials such as the synthetic magainin analog MSI-78, for which a phase III study for the topical treatment of diabetic foot ulcers has been completed (cited in [3]). It is suggested that antimicrobial peptides may be particularly useful in the future in the treatment of cutaneous infections.

The protozoon and enteric pathogen *Entamoeba histolytica* produces pore-forming peptides designated amoebapores. Three isoforms exist in the cytoplasmic granules of the amoeba [4] which all consists of 77-amino acid residues. The amoebapores has been predicted to fold into four amphipathic α-helices and to be stabilized by three disulfide bonds [5, 6]. The peptides kill metabolically active eukaryotic cells as well as bacteria by permeating their cytoplasmic membranes, and hence amoebae may use their pore-forming peptides as effector molecules to kill a variety of host cells in a contact-dependent reaction and also to prevent growth of engulfed bacteria inside their digestive vacuoles [7].

Amoebapores functionally resemble the defensins, antibacterial and cytotoxic peptides found in granules of mammalian neutrophils [8]. Whereas the β-stranded

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defensins are structurally clearly different, a polypeptide very similar to amoebapores was found with NK-lysin of porcine cytotoxic lymphocytes [9]. NK-lysin resembles amoebapores in functional and structural aspects [10]: the peptide displays cytotoxic and antibacterial activity but is non-hemolytic, it is of similar size, it shows substantial sequence similarity and, in particular, it possesses the same disulfide connectivities. Interestingly, with granulysin another structural correlate was found in human lymphocytes [11] and is currently viewed as an important constituent of the antimicrobial defense against intracellular pathogens [12].

Cytotoxic and antibacterial activity of amoebapores was found to be retained in the third helical region [13]. In a preceding study [14], various synthetic peptides were designed according to that structural element of each amoebapore isoforms. We examined the activity of the peptides towards artificial membranes, bacteria, and eukaryotic cells and found that some were even more antibacterial and/or cytotoxic than the parent molecules. The most promising model peptide for a pharmaceutical application was termed C1 and represented the putative helix 3 of amoebapore C. Despite its poor solubility, C1 displayed extraordinary pore-forming activity, exerted activity against bacteria, but was of low cytotoxicity [14].

In the present study, we examined the candidacidal activity of the synthetic amoebapore analogs constructed so far by determining their minimal growth inhibiting and lethal concentrations against a clinical isolate of *Candida albicans*, an important and widespread human pathogen. In addition, peptide C1 was used as a lead for the design of four novel peptides, in which sequence variations were made to enhance solubility and antimicrobial potency. Moreover, we constructed two synthetic peptides which were based on a highly cationic core region of NK-lysin that corresponds in sequence alignment to the helix 3 region of amoebapores [10]. Assuming a potential topical application to cutaneous infections, the most active peptides were tested for activity towards a human keratinocyte cell line to estimate their cytotoxicity. To complete the analysis of their spectrum, antibacterial and hemolytic activities of the novel peptides were also determined.

Materials and methods

Chemicals

Trifluoroacetic acid (TFA) and acetonitrile, both of HPLC grade, were from Applied biosystems. 2-(N-morpholino)ethane-sulfonic acid (MES) and valinomycin were purchased from Sigma. All other chemicals were of analytical grade and were obtained from Merck, Darmstadt.

Peptides

Peptides H3, A1, A2, A3, A4, A5, A6, A7, A8, B1, B2, B3, C1, C2, and C3 were synthesized and purified as described [14]. The newly constructed synthetic peptides C4, C5, C6, C7, NK1(40–63), and NK2(39–65) were synthesized on commission by Affiniti Research Products, Mamhead, Exeter, UK by solid-phase techniques utilizing an Fmoc-based protecting group strategy. Peptides were obtained in purity grade > 70%, dissolved in 0.1% TFA and purified further by reversed-phase (RP) chromatography as described [14]. The purified peptides were repeatedly lyophilized to evaporate the acid and stored at −20 °C. Amoebapores were highly purified from *E. histolytica* HM-1:IMSS as described previously [4]. Melittin, in HPLC grade, was purchased as an synthetic peptide from Sigma and used without further purification.

Peptide analysis

Homogeneity of all synthetic peptides was confirmed by analytical RP-HPLC (HAIsil C18 column, 250 × 2.1 mm, Higgins Analytical) showing a single peak for each one. Amino acid compositions of the peptides were confirmed by amino acid analysis performed as described previously [15] and by ion spray mass spectrometry (performed by the manufacturer). Peptide concentrations were determined by measuring absorbance at 214 nm; the extinction coefficients were calculated [16] using the respective sequence information. Chemical cross-linking with dithiobis(succinimidyl-propionate) (DSP) and subsequent Tricine-SDS/PAGE were performed as previously described [14]. Circular dichroism experiments were performed using a JASCO J-600 spectropolarimeter and quartz cells with a 0.02-cm path length in 10 mM sodium phosphate buffer, pH 6.8 at 25 °C; the peptide concentration was 80 μM as determined by amino acid analysis. Mean hydrophobicities and mean hydrophobic moments of the synthetic peptides were calculated based on the normalized consensus scale proposed by Eisenberg et al. [17] using the MacDNASIS program (Hitachi Software Engineering Co.). Peptide sequences were represented as helical wheels according to Schiffer and Edmundson [18] using the Protean program (DNAStar Inc.). The NMR structure of NK-lysin (1NKL.brk) was presented by the WEBLAB VIEWER LITE program (Molecular Simulations Inc., San Diego, Calif.).

Assay for antifungal activity

Different strains of *C. albicans* (ATCC 10231, ATCC 10261, Sc 5314, and DSM 6659 obtained from B. Hube, Botanical Institute, University of Hamburg; and a clinical isolate from our institute) were cultivated on Sabouraud dextrose agar plates at 28 °C for 48 h. One to three colonies were suspended in Sabouraud bouillon (3% Sabouraud liquid medium; Oxoid, Unipath, Wesel, Germany) to give a concentration of 3×10^3–4×10^3 colony-forming units (CFU)/ml. Subsequently, the fungal suspension was diluted fivefold with 10 mM sodium phosphate buffer pH 5.2. Peptides were dissolved in 0.01% TFA and twofold serial diluted in 10 mM sodium phosphate buffer, pH 5.2, in a microtitre plate. The fungal suspension (50 μl; approximately 30–40 CFU) was added to the same volume of peptide solution and the plates were incubated at 28 °C for 48 h. The minimal inhibitory concentration (MIC) was defined here as the concentration of the highest dilution of peptides at which the growth of *C. albicans* was suppressed. Portions of the incubation mixtures were plated on Sabouraud dextrose agar plates for counting CFU. The minimal lethal concentration (MLC) was defined as the highest dilution of peptides at which no formation of colonies was observed. The values were expressed as the mean of at least two independent experiments, each performed in duplicates, with a divergence of not more than one dilution step. The peptide solvent (0.01% TFA) showed no effect on the fungal growth.

Assay for pore-forming activity

Determination of pore-forming activity of samples by monitoring the dissipation of a valinomycin-induced diffusion potential in liposomes has been described previously in detail [19].

Assay for antibacterial activity

The bacterial strains used were *Bacillus subtilis* (strain 60015) and *Escherichia coli* K-12 (ATCC 23716) from the Botanical Institute, University of Hamburg; *E. coli* K-12 D31 [20]; and a clinical isolate of *Staphylococcus aureus* obtained at our institute. Bacteria were usually grown overnight in Luria-Bertani (LB) medium with constant shaking at 37 °C and subsequently inoculated in the same medium to reach the mid-logarithmic phase. *B. subtilis* was dispersed on LB-agar plates containing 1% glucose, grown overnight and subsequently inoculated in LB medium. Antibacterial activity was measured by a microdilution susceptibility test as described previously in detail [14]. Peptides were dissolved and twofold serially diluted in 10 mM sodium phosphate, pH 5.2, a bacterial suspension was added to the peptide solution, and the mixture was incubated at 37 °C. The MIC and MLC values were defined here as the concentration of the highest dilution of peptides at which the growth of bacteria was suppressed and at which no formation of bacterial colonies was observed after plating out the incubation mixture, respectively. The values were expressed as the mean of the results of at least two experiments, each performed in duplicates, with a divergence of not more than one dilution step.

Assays for cytotoxic activity

Hemolytic activity

Freshly collected human erythrocytes were washed with phosphate-buffered saline (10 mM sodium phosphate, 2 mM potassium chloride, 138 mM sodium chloride, pH 7.4), and subsequently resuspended in MES-buffered saline (20 mM MES, 140 mM sodium chloride, pH 5.5, 5×10^8/ml). Subsequently, 20 μl of erythrocyte suspension were incubated with 80 μl of peptide solution in the same buffer for 30 min at 37 °C. As controls, erythrocytes were incubated with distilled water (maximal lysis) and buffer alone (spontaneous lysis). Lysis was determined by measuring the concentration of released hemoglobin at 412 nm. Percent lysis was defined as 100 × (experimental lysis – spontaneous lysis)/(maximal lysis – spontaneous lysis).

Cytotoxicity assay

Cells of the human keratinocyte cell line HaCaT [21] (courtesy of N. E. Fusenig, Deutsches Krebsforschungszentrum Heidelberg) were cultivated in DMEM medium (Sigma) supplemented with 5% fetal calf serum (Sigma), 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B at 37 °C in a humidified atmosphere with 5% CO_2. The cells were detached from the culture flasks by EDTA (0.05%) treatment for 20 min, followed by EDTA/trypsin (0.025%/0.05%) treatment at 37 °C for 2–5 min. For the test, cells were suspended in DMEM medium to a density of 10^5 cells/ml. This suspension (100 μl) was filled in each well of a sterile flat-bottom 96-well micrometer plate. After incubation at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h, the medium was removed. Peptides, dissolved in 0.01% TFA at different concentrations and tenfold diluted with DMEM medium, were added to the wells (100 μl each) and subsequently incubated for further 20 h. As controls, 0.01% TFA was diluted ten times in medium and added to wells loaded with cells (= 100% viability) and to wells without cells (= 0% viability). For determination of cell viability, 10 μl of WST-1 solution {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, Boehringer Mannheim, Germany} was added to each well and subsequently incubated for 1 h at 37 °C. Finally, the extinction was measured using a microtitre-plate reader at absorption and reference wavelengths of 450 and 630 nm, respectively. Cytotoxic activity of the peptides was expressed as percent of remaining viable cells compared to the controls.

Results

Candidacidal activity of peptides

The candidacidal activity of the 21 synthetic peptides and of the three natural amoebapores was tested against a clinical isolate of *C. albicans* (Table 1). Melittin from bee venom, known for its potent and unspecific membranolytic activity [22], served as a control. Two peptides, namely A4 and B1, were of poor solubility in the buffer used for this assay and thus their minimal growth inhibiting activity could not be determined. Whereas the natural amoebapores displayed no candidacidal activity, some of their short versions were considerably potent. In addition to the peptides A6, A7, and A8, which are hybrids composed of the third and part of the fourth helix of amoebapore A, the newly constructed peptides C5, C6, C7 were highly active. Also remarkably potent were the two peptides representing NK-lysin, i.e., NK1(40–63) and NK2(39–65) which were designed according to the amino acid sequence of the isolated natural polypeptide and to the slightly differing one deduced from a porcine cDNA sequence, respectively. The minimal peptide concentration needed to achieve growth inhibition (MIC) was also effective in killing the yeast (MLC), which is in agreement with the notion that the peptides efficiently act on the target cell membranes. Comparison of the structural parameters dictated by the primary structures of the peptides indicates that there is a clear correlation between the net charge and the fungicidal activity within a related group of peptides (Table 1). Particularly, in C4, C5, C6, and C7, stepwise exchange of positively charged lysine residues for negatively charged residues, i.e., glutamic acid and aspartic acid, resulted in peptides with increasing positive net charge, and coincidently the candidacidal activity was substantially enhanced. Notably, the substitutions also markedly increased the solubility of these peptides in biological buffers compared to peptide C1. The novel amoebapore substitution analogs and the extremely charged NK peptides show a relatively low hydrophobicity. Virtually all peptides are highly amphipathic as indicated by the quite similar hydrophobic moments.

The two most potent peptides C7 and NK2(39–65) were also tested against four *C. albicans* strains from culture collections. Despite the fact that these strains were marginally less susceptible than the clinical isolate tested before, these peptides again were highly effective and displayed candidacidal activity comparable to melittin (Table 2).

Other properties of the newly designed peptides

To complete the activity spectrum of the newly designed peptides, we examined their antibacterial (Table 3), pore-forming (Fig. 1), and hemolytic (Fig. 2) activities. The extraordinary pore-forming activity of C1 (3.7 ± 1.2 U/pmol [14]) was not found again among the C1-

Table 1 Activity of membrane-active peptides against a clinical isolate of *Candida albicans*. Peptides designated as A, B, and C are derived from amoebapore A, B, and C, respectively. The C termini of all synthetic peptides are amidated with the exception of that of H3, the prototype of the structural amoebapore analogs. Net charges of the peptides were calculated by counting the N terminus, arginine, lysine, and histidine as positive charges and a free C terminus, aspartic acid, and glutamic acid as negative charges. H and μ were calculated for the entire peptide according to Eisenberg [17]. A point (•) marks the omission of an amino acid residue. A hyphen indicates the identical amino acid residue as in the respective sequence of H3, B1, or C1. Peptides A1 and C1 were soluble in the buffer used only up to a concentration of 5 μM (*MIC* minimal growth inhibitory concentration, *MLC* minimal lethal concentration, *H* mean hydrophobicity, μ mean hydrophobic moment, *n.d.* not determined since peptides A4 and B1 were not soluble in the buffer used for this assay, > no inhibition found at the concentration indicated)

Peptide	Sequence	MIC (μM)	MLC (μM)	Net charge	H	μ
H3	GFIATLCT**K**VLDFGID**K**LIQLIED**K**	>10	>10	−1	0.25	0.28
A1	----------	>5	>5	−1	0.32	0.35
A2	----------	>10	>10	0	0.25	0.28
A3	------•----------	2.5	2.5	0	0.25	0.38
A4	------L----------	n.d.	n.d.	−1	0.35	0.38
A5	------L----------**K**	2.5	2.5	+1	0.33	0.39
A6	------L----------**KIH**	1.3	1.3	+3	0.34	0.21
A7	------L----------**KIH**	0.6	0.6	+3	0.31	0.31
A8	------L----------**KIH**A	0.6	0.6	+3	0.32	0.28
B1	GFLGTLCE**K**ILSFGVDELV**K**LIEN	n.d.	n.d.	−1	0.30	0.34
B2	----------**H**	10	10	0	0.27	0.31
B3	------•----------**H**	2.5	2.5	0	0.27	0.41
C1	GLVETLCT**K**IVSYGID**K**LIE**K**ILE	>5	>5	0	0.23	0.25
C2	----------G	>10	>10	0	0.24	0.25
C3	------•----------G	10	10	0	0.24	0.42
C4	----------**K**--------	1.3	1.3	+2	0.21	0.26
C5	---**K**----------**K**--------	0.6	0.6	+4	0.17	0.23
C6	---**K**----------**K**----	0.6	0.6	+4	0.17	0.25
C7	---**K**----------**K**---**K**----	0.15	0.3	+6	0.14	0.26
NK1(40–63)	IL**R**GLC**KK**IM**R**SFL**RR**ISWDILTG	0.6	0.6	+6	−0.03	0.30
NK2(39–65)	**K**IL**R**GVC**KK**IM**R**TFL**RR**IS**K**DILTG**KK**	0.15	0.15	+10	−0.28	0.42
Melittin	GIGAIL**K**VLATGLPTLISWI**K**N**KRK**Q	0.6	0.6	+6	0.15	0.34
Amoebapore A		>10	>10	0		
Amoebapore B		>10	>10	−1		
Amoebapore C		>10	>10	−1		

Table 2 Activity against four different strains of *C. albicans*. Candidacidal activity was determined at least in two independent experiments with a discrepancy not more than one dilution step (*MIC* minimal inhibitory concentration, *MLC* minimal lethal concentration)

C. albicans strain	MIC (MLC), μM		
	C7	NK2(39–65)	Melittin
ATCC 10231	0.3 (0.3)	0.3 (0.3)	0.6 (0.6)
ATCC 10261	0.6 (0.6)	1.3 (1.3)	1.3 (1.3)
Sc 5314	0.6 (0.6)	0.6 (0.6)	1.3 (1.3)
DSM 6659	0.6 (0.6)	0.6 (0.6)	0.6 (0.6)

Table 3 Antibacterial activity of synthetic peptides. Antibacterial activity was determined at least in two independent experiments with a discrepancy not more than one dilution step and is expressed as MIC (MLC) in μM (> no inhibition found at the concentration indicated)

Peptide	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli* K-12	*Escherichia coli* K-12 D31
C4	10 (10)	>10 (>10)	10 (10)	5 (5)
C5	0.6 (0.6)	5 (5)	0.6 (0.6)	0.3 (0.3)
C6	0.6 (0.6)	5 (5)	1.3 (1.3)	0.3 (0.3)
C7	0.6 (0.6)	2.5 (5)	0.6 (1.3)	0.15 (0.3)
NK1(40–63)	1.3 (1.3)	2.5 (5)	1.3 (1.3)	0.3 (0.3)
NK2(39–65)	0.6 (0.6)	2.5 (5)	0.6 (0.6)	0.15 (0.15)
Melittin	0.6 (1.3)	0.6 (2.5)	0.6 (2.5)	0.6 (0.6)

based peptides as they exhibited a pronounced, but significantly lower activity than the lead peptide C1. Their antibacterial activity, however, was found to be markedly increased by the substitutions irrespective whether the targets were Gram positive or Gram negative, and even the more resistant clinical isolate of *S. aureus* was effectively killed. In contrast to C1, the peptides derived thereof were quite hemolytic. Remarkably, the NK2(39–65) were virtually non-hemolytic and herein resembles its parent molecule NK-lysin.

The majority of the active peptides contains a free sulfhydryl group, and dimerization of these peptides upon disulfide bond formation may enhance their efficacy on target cell membranes. Association with liposomes and chemical cross-linking with DSP yielded dimers, tetramers and sometimes even higher molecular mass entities (data not shown), suggesting that the dimer serves as a nucleation point for further oligomerization as already found with other peptides containing a sole cysteine residue [14, 23].

Cytotoxic activity towards human skin cells

To estimate their therapeutic potential, peptides with significant candidacidal activity were assayed for cyto-

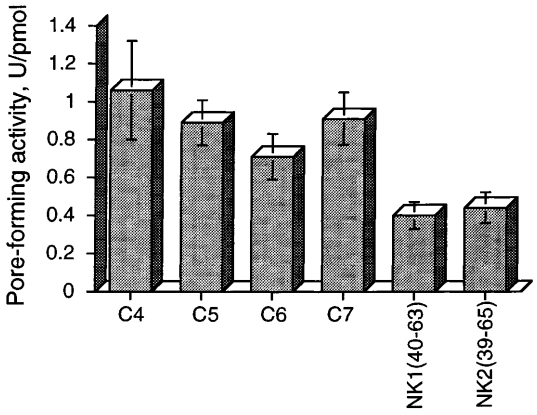

Fig. 1 Pore-forming activity of synthetic peptides. Pore-forming activity was measured by liposome depolarization and determined in at least eight independent experiments. Activity is expressed in U/pmol (mean ± SD)

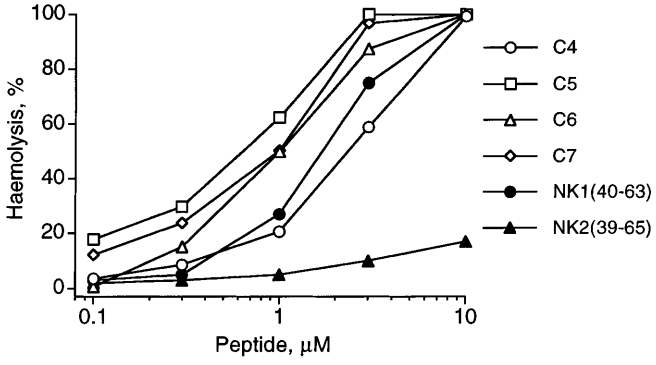

Fig. 2 Hemolytic activity of synthetic peptides. Hemolytic activity was determined by measuring the release of hemoglobin after incubation of the peptides with human erythrocytes for 30 min at 37 °C. Values were determined at least in two independent experiments, each performed in duplicates

toxicity towards the human keratinocyte cell line HaCaT (Fig. 3). In this assay, the activity of mitochondrial dehydrogenases of the cells was determined by measuring the formation of formazan by reduction of a tetrazolium salt (WST-1). The enzyme activity is a measure of cell proliferation and viability. Here, peptides A5, A6, A7, and A8 were cytotoxic in concentrations of their antimicrobial efficacy. In contrast, peptides C4, C5, C6, and the NK peptides had no or only little effect towards these cells in the concentration range tested.

Structural analysis of NK2(39–65)

Preliminary experiments using circular dichroism spectroscopy had shown that several of the peptides are mainly α-helical or have a high potential to adopt an α-helical conformation in anisotropic media such as 50% trifluoroethanol (data not shown). For a more detailed analysis, NK2(39–65) was chosen because of its potent antimicrobial activity combined with low hemolytic and

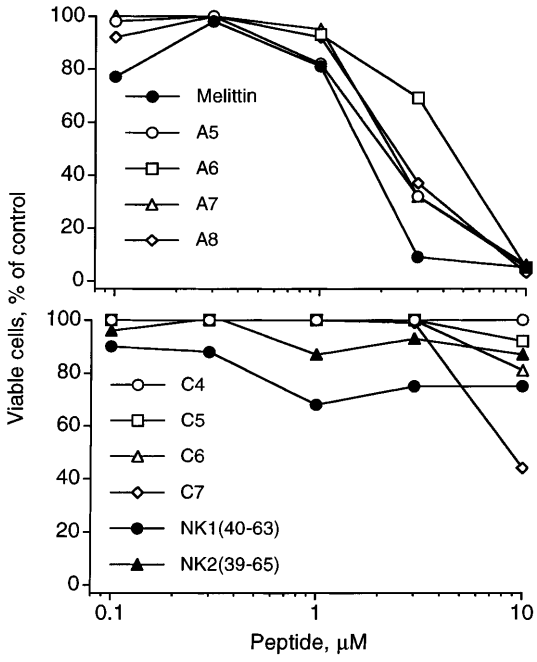

Fig. 3 Cytotoxicity of synthetic peptides against HaCaT cells. Cell death after exposure to various concentrations of peptides is expressed as percent of remaining viable cells compared to the control of untreated cells. Viability of cells was determined photometrically by measuring their ability to reduce the tetrazonium salt WST-1. The activity was determined in two independent experiments at least in sextuples

cytotoxic activity (Fig. 4). The spectra revealed that in phosphate buffer the peptide is mainly unstructured, whereas it became all-α-helical in the presence of 0.4% SDS or 50% trifluoroethanol. Quantitative analysis of the spectra using the Contin program [24] gave the following values for the content of α-helix, β-sheet and remainder, respectively: 11%, 11%, and 78% in buffer; 91%, 0%, and 9% in SDS; 100%, 0%, and 0% in trifluoroethanol. The helical wheel diagram (Fig. 4) revealed the amphipathic nature of the peptide, i.e., the clear segregation of hydrophilic and hydrophobic residues on opposite faces of the helix. Moreover, a very large angle subtended by the positively charged residues became apparent.

Discussion

Natural antimicrobial peptides have been the lead substances for the design of a variety of synthetic peptides with optimized activity [3]. By altering their sequence, attempts have been made to increase antimicrobial activity but to maintain or even decrease a low activity against host cells. In the present study, the templates were corresponding core regions of larger natural polypeptides which are structurally and functionally similar, namely amoebapores and NK-lysin. It was found that the target spectrum of short versions of amoebapores with antibacterial potency may be extended towards the

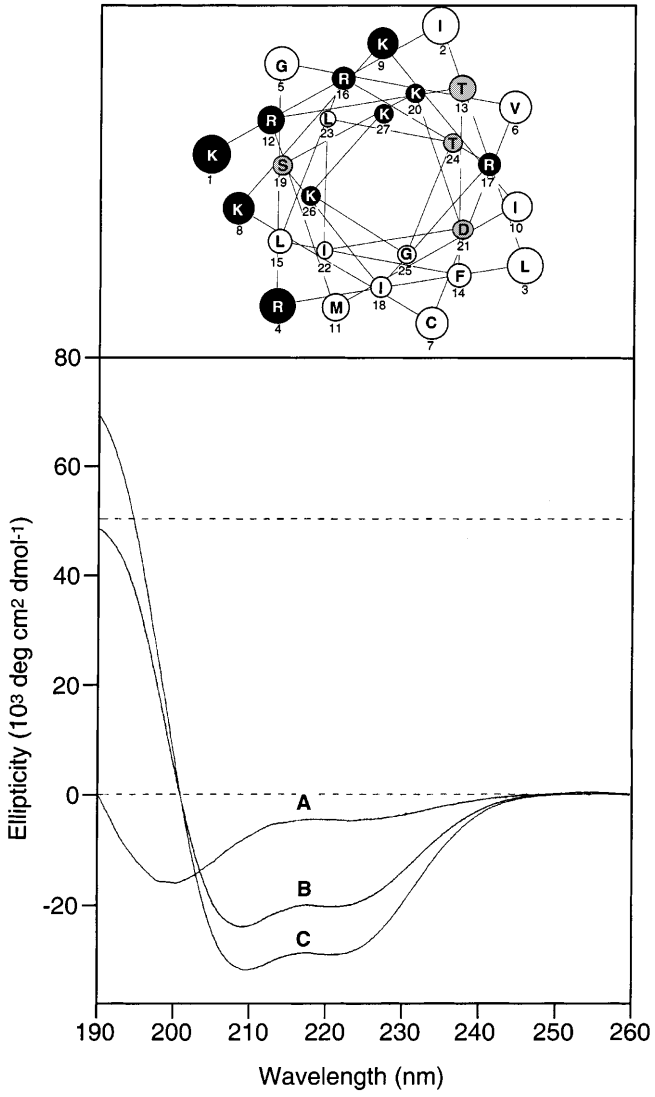

Fig. 4 Helical wheel diagram and circular dichroism analysis of NK2(39–65). *Upper panel* Helical wheel diagram of peptide NK2(39–65). Positively charged residues are presented as *filled circles*, residual hydrophilic residues as *shaded circles*, and hydrophobic residues as *open circles*. Hydrophobicity was determined according to the consensus scale of Eisenberg [17]. *Lower panel* Circular dichroism spectra of NK2(39–65) in buffer alone (*A*), in the presence of 0.4% SDS (*B*), and in the presence of 50% trifluoroethanol (*C*)

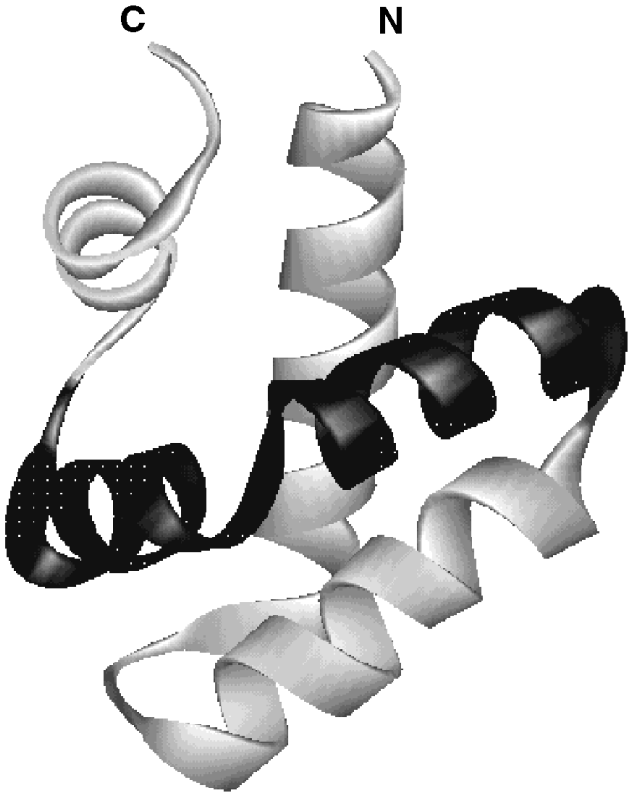

Fig. 5 Position of the region corresponding to NK2(39–65) within natural NK-lysin. The three-dimensional structure of NK-lysin as solved by NMR (1NKL.brk) is presented by the WEBLAB VIEWER LITE program. The *dark region* marks the part of the structure that resembles the synthetic peptide deduced from the NK-lysin sequence. Disulfide bonds are omitted

fungal pathogen *C. albicans*. Antimicrobial activity of amoebapore-deduced peptides that represent the putative helix 3 of amoebapore C were gradually improved by substitution of negatively charged residues by lysine residues. With the NK-lysin-derived peptides, we isolated a region from the entire molecule that corresponds to the putative helix 3 of amoebapores and we thereby apparently separated the antimicrobial activities of NK-lysin from the cytotoxic potency also inherent in the natural protein. Especially NK2(39–65) has several properties favorable for a broad-spectrum antibiotic peptide: it exerts potent candidacidal activity, displays antibacterial activity towards Gram-positive and Gram-negative bacteria, is virtually non-hemolytic and is non-toxic to human skin cells. The very large angle subtended by the positively charged helix face of the latter peptide may be suitable for binding to microbes, and particularly this structural parameter has been considered important for target selectivity [25]. The accompanying low hydrophobicity may reduce cytotoxicity [26].

As the structure of natural NK-lysin was recently solved by NMR (1NKL.brk) [27], the location of the region to which the synthetic NK-peptides correspond can be located within the structure of the entire protein. The region envisaged as a long helix at time when the peptides were designed actually harbors two (sub)helices, i.e., helix 3 and 4. The longer peptide NK2(39–65) comprises in addition to the two tightly connected helices the short connectivities to adjacent helices (Fig. 5). It was experimentally proven that this peptide adopts an all-α-helical conformation in media simulating a membrane environment.

It may be assumed that most of the active peptides studied here can be classified best as linear amphipathic α-helical peptides, and hence are comparable to well-known members of the largest among the groups of antimicrobial peptides such as cecropins, magainins, and dermaseptins [28]. Significantly, in addition to their mostly α-helical structure naturally occurring antimi-

crobial peptides are cationic in nature. The most active synthetic peptides studied here are cationic and some of them, in particular NK2(39–65), show a 'rhythm' of positively charged residues which is reminiscent again of cecropins, magainins, and dermaseptins [28–30]. It was found that a high number of positive charges within antimicrobial peptides allow selective binding to microbial targets in that they primarily promote binding to negatively charged surfaces [31]. Interestingly, amoebapores – and also the unsubstituted helical elements thereof – are, despite their antibacterial activity, not effective against *Candida*. This is contrast to the potency of NK-lysin [9] and is most likely due to the substantially lower amount of positive charges on the surface of the amoebic polypeptides [32].

The MIC values of all the synthetic peptides for their activity towards *C. albicans* were identical or very close to the their MLC values, excluding an entirely fungistatic activity. Likewise, growth inhibition of bacteria by these peptides was due to killing of bacteria. Considering that all the peptides were capable of rendering liposomes permeable, one may suppose that the cytoplasmic membrane is their target. Despite the increasing number of models that describe the proposed mechanism by which small peptides permeabilize membranes [33–37], membrane permeation of amphipathic α-helical peptides may presumably be divided into two mechanisms (reviewed in [38]): (a) the formation of a transmembrane pore via the "barrel-stave mechanism" by which the peptides insert, are oriented perpendicular to the plane of the bilayer and upon oligomerization create a water-filled channel; and (b) the destruction of the membrane via the "carpet mechanism", by which the peptides bind onto the surface of the target until at least a part of it is covered and permeabilization takes place only after a threshold concentration is reached. The aforementioned resemblance to natural linear α-helical peptides may have implications as to the mode of action of the synthetic peptides presented here, and hence it is much more likely that they act according to the second mechanism as described for cecropin P1 [39].

Resistance of fungal pathogens to classical antibiotics is currently not as commonly found as with bacteria but has become an increasing problem [40]. Antimicrobial peptides designed according to natural templates as those presented here may provide lead substances for the development of peptide antibiotics which may be used alone or in combination with classical antibiotics in selected pathologies induced by pathogenic or opportunistic microorganisms.

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References

1. Boman HG, Marsh J, Goode JA (eds) (1994) Antimicrobial peptides, Ciba Found Symp 186. Wiley, Chichester
2. Hancock REW, Lehrer R (1998) Cationic peptides: a new source of antibiotics. TIBTECH 16:82–88
3. Hancock REW (1997) Peptide antibiotics. Lancet 349:418–422
4. Leippe M, Andrä J, Nickel R, Tannich E, Müller-Eberhard HJ (1994) Amoebapores, a family of membranolytic peptides from cytoplasmic granules of *Entamoeba histolytica*: isolation, primary structure, and pore formation in bacterial cytoplasmic membranes. Mol Microbiol 14:895–904
5. Leippe M, Tannich E, Nickel R, Goot G v d, Pattus F, Horstmann RD, Müller-Eberhard HJ (1992) Primary and secondary structure of the pore-forming peptide of pathogenic *Entamoeba histolytica*. EMBO J 11:3501–3506
6. Dandekar T, Leippe M (1997) Molecular modeling of amoebapore and NK-lysin: a four-alpha-helix bundle motif of cytolytic peptides from distantly related organisms. Folding Design 2:47–52
7. Leippe M (1997) Amoebapores. Parasitol Today 13:178–183
8. Lehrer RI, Lichtenstein AL, Ganz T (1993) Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu Rev Immunol 11:105–128
9. Andersson M, Gunne H, Agerberth B, Boman A, Bergman T, Sillard R, Jörnvall H, Mutt V, Olsson B, Wigzell H, Dagerlind A, Boman HG, Gudmundsson GH (1995) NK-Lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity. EMBO J 14:1615–1625
10. Leippe M (1995) Ancient weapons: NK-lysin is a mammalian homolog to pore-forming peptides of a protozoan parasite. Cell 83:17–18
11. Peña SV, Hanson DA, Carr BA, Goralski TJ, Krensky AM (1997) Processing, subcellular localization, and function of 519 (Granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. J Immunol 158:2680–2688
12. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T, Thoma-Uszynski S, Melian A, Bogdan C, Porcelli SA, Bloom BR, Krensky AM, Modlin RL (1998) An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 282:121–125
13. Leippe M, Andrä J, Müller-Eberhard HJ (1994) Cytolytic and antibacterial activity of synthetic peptides derived from amoebapore, the pore-forming peptide of *Entamoeba histolytica*. Proc Natl Acad Sci USA 91:2602–2606
14. Andrä J, Berninghausen O, Wülfken J, Leippe M (1996) Shortened amoebapore analogs with enhanced antibacterial and cytolytic activity. FEBS Lett 385:96–100
15. Andrä J, Leippe M (1994) Pore-forming peptide of *Entamoeba histolytica*: significance of positively charged amino acid residues for its mode of action. FEBS Lett 354:97–102
16. Buck MA, Olah TA, Weitzmann CJ, Cooperman BS (1989) Protein estimation by the product of integrated peak area and flow rate. Anal Biochem 182:295–299
17. Eisenberg D, Schwarz E, Komaroy M, Wall R (1984) Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J Mol Biol 179:125–142
18. Schiffer M, Edmundson AB (1967) Use of helical wheels to represent the structure of proteins and to identify segments with helical potential. Biophys J 7:121–135
19. Leippe M, Ebel S, Schoenberger OL, Horstmann RD, Müller-Eberhard HJ (1991) Pore-forming peptide of pathogenic *Entamoeba histolytica*. Proc Natl Acad Sci USA 88:7659–7663
20. Boman HG, Nilsson-Faye I, Paul K, Rasmuson TJ (1974) Insect immunity. I. Characteristics of an inducible cell-free antibacterial reaction in hemolymph of *Samia cynthia* pupae. Infect Immun 10:136–145
21. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE (1988) Normal keratinization in a

spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106:761–771
22. Dempsey CE (1990) The action of melittin on membranes. Biochim Biophys Acta 1031:143–161
23. Takei J, Remenyi A, Dempsey CE (1999) Generalised bilayer perturbation from helix dimerisation at membrane surfaces: vesicle lysis induced by disulphide-dimerised melittin analogues. FEBS Lett 442:11–14
24. Provencher SW, Glöckner J (1981) Estimation of globular protein secondary structure from circular dichroism. Biochemistry 20:33–37
25. Wieprecht T, Dathe M, Epand RM, Beyermann M, Krause E, Maloy WL, MacDonald DL, Bienert M (1997) Influence of the angle subtented by the positively charged helix face on the membrane activity of amphipathic, antibacterial peptides. Biochemistry 36:12869–12880
26. Dathe M, Wieprecht T, Nikolenko H, Handel L, Maloy WL, MacDonald DL, Beyermann M, Bienert M (1997) Hydrophobicity, hydrophobic moment and angle subtented by charged residues modulate antibacterial and hemolytic activity of amphipathic helical peptides. FEBS Lett 403:208–212
27. Liepinsh E, Andersson M, Ruysschaert J-M, Otting G (1997) Saposin fold revealed by the NMR structure of NK-lysin. Nat Struct Biol 4:793–795
28. Boman HG (1995) Peptide antibiotics and their role in innate immunity. Annu Rev Immunol 13:61–92
29. Maloy WL, Kari UP (1995) Structure-activity studies on magainins and other host defense peptides. Biopolymers 37:105–122
30. Nicolas P, Mor A (1995) Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu Rev Microbiol 49:277–304
31. Matsuzaki K, Sugishita K, Fujii N, Miyajima K (1995) Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. Biochemistry 34:3423–3429
32. Bruhn H, Leippe M (1999) Comparative modeling of amoebapores and granulysin based on the NK-lysin structure – structural and functional implications. Biol Chem 380:1001–1007
33. Boheim GJ (1974) Statistical analysis of alamethicin channels black lipid membranes. J Membr Biol 19:277–303
34. Shai Y (1995) Molecular recognition between membrane-spanning polypeptides. Trends Biochem Sci 20:460–464
35. Ludtke SJ, He K, Heller WT, Harroun TA, Yang L, Huang HW (1996) Membrane pores induced by magainin. Biochemistry 35:13723–13728
36. Matzusaki K (1998) Magainins as paradigm for the mode of action of pore forming polypeptides. Biochim Biophys Acta 1376:391–400
37. Wu M, Maier E, Benz R, Hancock RE (1999) Mechanism of interaction of different classes of cationic peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. Biochemistry 38:7235–7242
38. Oren Z, Shai Y (1998) Mode of action of linear amphipathic alpha-helical antimicrobial peptides. Biopolymers (Peptide Sci) 47:451–463
39. Gazit E, Boman A, Boman HG, Shai Y (1995) Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles. Biochemistry 34:11479–11488
40. Wout JW van' t (1996) Fungal infection and antifungal drugs: has the age of antifungal resistance dawned. Curr Opin Infect Dis 9:63–66