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N-terminal fatty acylation of peptides spanning the cationic C-terminal segment of bovine β-defensin-2 results in salt-resistant antibacterial activity

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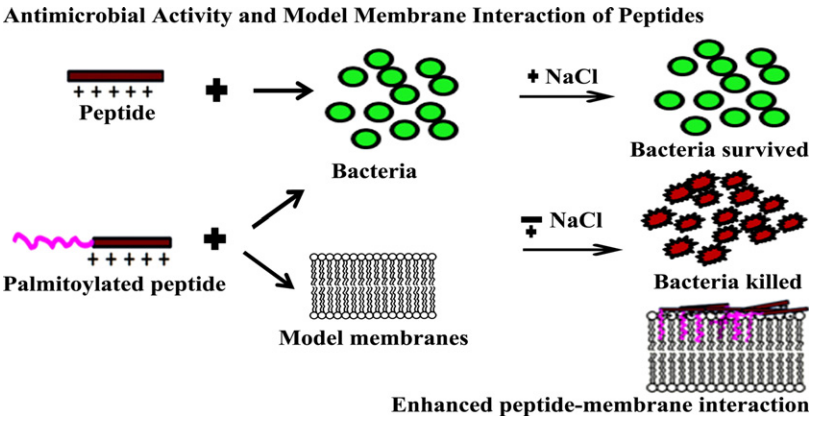
HIGHLIGHTS

- Investigated antimicrobial activity of N-acylated defensin analogs.
- Both acetylated and palmitoylated peptides exhibited antimicrobial activity.
- Palmitoylated peptides showed antibacterial activity in the presence of salt.
- Palmitoylation resulted in enhanced binding to model membranes.
- Observed hemolytic activity is reduced in the presence of lipid vesicles and serum.

GRAPHICAL ABSTRACT



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ABSTRACT

Peptides spanning the C-terminal segment of bovine-β-defensin-2 (BNBD-2) rich in cationic amino acids, show antimicrobial activity. However, they exhibit considerably reduced activity at physiological concentration of NaCl. In the present study, we have investigated whether N-terminal acylation (acetylation and palmitoylation) of these peptides would result in improved antimicrobial activity. N-terminal palmitoylation though increased hydrophobicity of the peptides, did not enhance antimicrobial potency. However, antibacterial activity of these peptides was not attenuated by NaCl. Biophysical studies on the palmitoylated peptides have indicated that antibacterial activity in the presence of NaCl arises due to the ability of the peptides to interact with membranes more effectively. These peptides showed hemolytic activity which was attenuated considerably in the presence of serum and lipid vesicles. In defensin related peptides, fatty acylation would be a convenient way to generate analogs that are active in the presence of salt.

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1. Introduction

Defensins are important components of host-defense against bacteria and fungi. They are present in species across the evolutionary scale [1–6]. The primary structures of defensins from several mammalian species have been determined [5–9]. Defensins, particularly in mammals, are characterized by three disulfide bonds and based on the disulfide connectivity, they are referred to as α or β defensins [5–9]. Apart from the disulfide pattern and certain conserved residues, there are considerable variations in the net positive charge (at neutral pH) and peptide chain length. The antimicrobial spectrum and sensitivity of antimicrobial activity to salt also vary considerably [10–12]. Extensive efforts have been made to correlate the structural and sequence requirements for activity [13–20]. From these studies, it emerges that the native disulfide connectivity or even three disulfides are not essential for antimicrobial activity [14,17]. The cationic residues and the presence of certain amino acids appear to play a critical role in determining the activity of native defensins [14,17]. Linearized defensin analogs exhibit antibacterial activity although they do not adopt amphipathic helical structures [21]. Some native defensins tend to form dimers in solution [22,23]. This structural feature does not appear to be essential for exhibiting activity as peptides with non-native disulfide bridges as well as linear analogs that are unlikely to oligomerize, also exhibit activity [22,23]. Peptides spanning the cationic segments of β-defensins with a single disulfide bridge show antimicrobial activity indicating that there is considerable flexibility in the sequence and structural requirement for antimicrobial activity [14]. However, the antibacterial activities of the truncated defensin analogs are attenuated in the presence of high concentrations of salt. Several attempts have been made to design analogs of defensins whose antimicrobial activities are not affected by NaCl. These approaches have involved synthesis of hybrid defensins or other synthetic modifications [24,25]. We have earlier shown the peptides spanning the C-terminal segment of bovine β-defensin BNBD-2 have antibacterial activity. However, these peptides were inactive in the presence of high NaCl concentrations [14]. In this study, we have explored whether salt resistant defensin analogs can be obtained with minimal synthetic intervention.

Fatty acid acylation is a common post-translational modification occurring in a wide variety of eukaryotic proteins and has an important role in intracellular protein sorting and targeting to membranes [26]. Attachment of fatty acid to a protein or peptide would result in increased hydrophobicity and favor greater membrane interaction [26]. Studies on short acylated antimicrobial peptides indicate that addition of a fatty acid can modulate, both activity and selectivity [27–36]. For example, in the case of polymyxin, removal of the fatty acid resulted in reduced antimicrobial potency, indicating that it is important for activity [37]. The attachment of palmitic acid to the N-terminus of a positively charged model peptide, eliminated its antibacterial activity but increased its antifungal and hemolytic activity [28]. Correlation between secondary structure induced by lipids and antibacterial or hemolytic activity has been observed in fatty acylated peptides [28,31]. Studies on fatty acylated mastoparan have indicated that embedding and positioning of the peptide in the membrane modify its effective charge which plays a critical role in the selectivity of peptide between neutral and negatively charged lipid membranes [29]. In the present study, the C-terminal segment RCPGRTRQIGTIFGPRIKCRSW (PB1) of BNBD-2 (sequence shown in Table 1), and its variant (PB4) have been modified with acetyl group or palmitic acid at the N-terminus. The antimicrobial activity of the modified peptides (Table 1) was determined in the presence and absence of NaCl. Their interaction with model membranes was also investigated.

Table 1
Sequences of bovine β-defensin BNBD-2 and its analogs.

VRNHVTC$_1$RINRGFC$_2$VPIRC$_3$PGRTRQIGTC$_4$FGPRIKC$_5$C$_6$RSW	BNBD-2
RCPGRTRQIGTIFGPRIKCRSW PB1	(PB1L)[a]
RC^{D}PGRTRQIGTIF^{D}PGRIKCRSW PB4	(PB4L)[a]
Palmitoyl-RCPGRTRQIGTIFGPRIKCRSW PA1	(PA1L)
Palmitoyl-RC^{D}PGRTRQIGTIF^{D}PGRIKCRSW	PA4 (PA4L)
Acetyl-RCPGRTRQIGTIFGPRIKCRSW	AC1 (AC1L)
Acetyl-RC^{D}PGRTRQIGTIF^{D}PGRIKCRSW	AC4 (AC4L)

Disulfide linkages are between C_1–C_5, C_2–C_4 and C_3–C_6 in BNBD-2.
^{D}P indicates D-proline.
Peptides PA1L, PA4L, AC1L and AC4L (in parentheses) are linear forms of peptides where the cysteine side chain protecting acetamidomethyl (acm) group has been retained.
[a] Peptide sequences corresponding to C-terminal segment of BNBD-2 [14].

2. Materials and methods

2.1. Materials

Fluorenylmethoxycarbonyl (F-moc) protected amino acids used were from Novabiochem AG, Switzerland and Advanced Chemtech, Louisville, KY, USA. Coupling reagents used in peptide synthesis were N-hydroxybenzotriazole hydrate (HOBt) (Advanced Chemtech), 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Advanced Chemtech), and N, N-diisopropylethylamine (DIPEA) (Sigma). Palmitic acid used for acylation of peptides was from Sigma-Aldrich, MO, USA. POPC, 1-palmitoyl-2-oleoyl-*sn*-glycerophospho-choline, POPE, 1-palmitoyl-2-oleoyl-*sn*-glycerophospho-ethanolamine and POPG, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoglycerol were purchased from Avanti Polar Lipids (Alabaster). Cholesterol was obtained from Sigma. All reagents and solvents used were of highest purity available.

2.2. Peptide synthesis

The synthesis of resin-bound C-terminal segment of BNBD-2 peptides with free amine group at the N-terminus has been described earlier [14]. The peptides were synthesized on HMPA resin using Fmoc chemistry [14]. N-terminal palmitoylation or acetylation of these peptides was carried out on-resin using the method described elsewhere [38,39]. N-terminal palmitoylated peptides were obtained by adding the mixture containing palmitic acid: HOBt: HBTU: DIPEA (1:1:1:2) to the resin-bound C-terminal segment of BNBD-2 and kept on gentle mixing for 5 h. The process was repeated two to three times and washed the resin thoroughly with DMF and diethyl ether. A mixture of acetic anhydride and DIPEA (1:1) was added to the resin and kept on gentle mixing for 1 h. The process was repeated two to three times and washed the resin thoroughly with DMF and diethyl ether to obtain N-terminal acetylated peptides. Peptides were cleaved from the resin using trifluoroacetic acid containing thioanisole, *m*-cresol, and ethanedithiol (10:1:1:0.5 v/v). The side chain protecting group in cysteines, acetamidomethyl (acm) was removed by treatment with mercuric acetate as described earlier [40]. Formation of disulfide bond was accomplished by oxidation in 20% aqueous dimethyl sulfoxide at a concentration of 0.5 mg/ml for 24 h at room temperature [14,41]. Peptides were purified by HPLC on a reversed phase C-18 column (Agilent Extent C-18 (4.6 mm × 250 mm)) using gradients of solvents: (A) 0.1% TFA in water; (B) 0.1% TFA in acetonitrile. The adsorbed peptides were eluted with a linear gradient of (10–70% solvent B) in 65 min at a flow rate of 0.5 ml/min. The purity of HPLC-purified peptides was ≥98%. The molecular masses of HPLC purified peptides were confirmed on an electrospray ionization mass spectrometer with a linear ion trap mass analyzer (LTQ-IT, Thermo Fischer, Waltham, MA, USA).

2.3. Antimicrobial activity

Antibacterial activity of the peptides against bacterial strains *Escherichia coli* (MG1655), *Staphylococcus aureus* (ATCC 8530) and

Pseudomonas aeruginosa (NCTC 6751) was determined as described previously [42,43]. Bacteria were grown in LB medium to mid-log phase and diluted to 10^6 colony-forming units (cfu)/ml in 10 mM sodium phosphate buffer pH 7.4 (PB). Bacteria were incubated with different concentration of peptides (1–15 μM) for 2 h at 37 °C, in sterile 96-well plates in a final volume of 100 μl. Suitably diluted aliquots were spread on LB agar plates. The plates were incubated at 37 °C for 18 h. The protocol is similar to the one used for defensins [42,43]. The cfu were counted, and percentage killing of bacteria relative to the cfu in untreated controls was calculated. The concentration of the peptides at which no viable colonies formed was taken as lethal concentration (LC), which was determined from the average of three independent experiments done in duplicates. Human serum (20%) or 150 mM NaCl was included in the incubation buffer to determine the effect of serum or salt.

The antifungal activity of peptides against *Candida albicans* (ATCC 18804) in final volumes of 100 μl was determined as described previously [44,45]. Minimum fungicidal concentrations (MFC) of the peptides were determined by growing *C. albicans* aerobically in yeast extract–peptone–dextrose (YEPD) medium at 30 °C. After 20 h, 0.3 ml from this suspension was subcultured for 2 h in 20 ml of YEPD broth to obtain a mid-log-phase culture. Cells were harvested by centrifugation, washed with PB and resuspended in the same buffer, and the concentration was adjusted to 10^6 cells/ml. Aliquots of diluted cells were incubated with different concentrations of peptides (1–15 μM) in 100 μl volumes at 30 °C for 2 h. Suitably diluted aliquots of cell suspensions were spread on YEPD agar plates and incubated for 24 h at 30 °C. Colonies were counted, and the concentrations of the peptides at which no viable colonies were formed were taken as the MFC. The averages of results from three independent experiments done in duplicates were taken for the calculation of MFC.

2.4. ANS fluorescence measurements

Fluorescence measurements were carried out on a Hitachi F-7000 Fluorescence spectrophotometer. ANS (4 μM) was titrated with increasing concentration of acylated peptide in 10 mM phosphate buffer pH 7.4 in the presence and absence of 150 mM NaCl. The samples were excited at 350 nm, and the emission spectrum was recorded from 420 to 550 nm. The excitation and emission slits were set to 5 nm. All measurements were carried out at 25 °C.

Table 2
Antimicrobial activity of acylated peptides and the analogs spanning the C-terminal segment of bovine β-defensin BNBD-2.

Peptide	LC (μM)[a]			MFC (μM)[a]
	E. coli	*P. aeruginosa*	*S. aureus*	*C. albicans*
PB1L	4[b]	5[b]	5[b]	7
PB1	4[b]	6[b]	4[b]	20
PB4L	4[b]	10[b]	6[b]	6
PB4	4[b]	5[b]	6[b]	20
PA1L	7	11	6	10
PA1	5	10	3	12
PA4L	5	12	4	12
PA4	5	10	2	12
AC1L	11	13	5	10
AC1	5	11	2	11
AC4L	5	12	5	8
AC4	5	12	2	10

Lethal concentration (LC). Minimum fungicidal concentrations (MFC).
[a] Variations in LC or MFC between independent experiments were ±1 μM.
[b] Indicates LC values taken from earlier report [14].

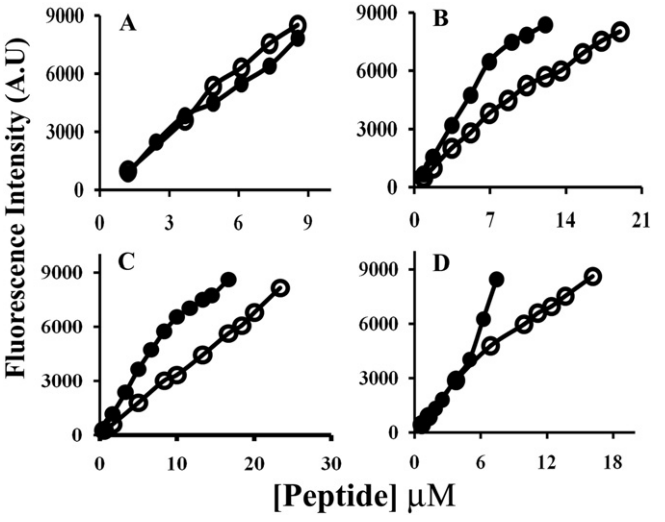

Fig. 1. Binding of ANS to linear and cyclic variants of N-terminal palmitoylated BNBD-2 analogs in the presence and absence of NaCl. Different concentrations of peptides were added to ANS (4 μM). The increase in fluorescence λ_{max} (470 nm) is plotted against peptide concentration. (A) PA1L, (B) PA1, (C) PA4L and (D) PA4. Key: (●) − NaCl, (○) + NaCl.

2.5. Circular dichroism (CD)

CD spectra were recorded on a Chirascan-plus CD spectrometer (Applied Photophysics, Leatherhead, UK). A resolution of one-nanometer and scan speed of 0.2 nm were applied to record far-UV spectra (190 nm to 260). Peptide concentrations of 10 μM were used to record the spectra in 10 mM phosphate buffer pH 7.4 in the presence of above critical micellar concentration (CMC) of SDS. Spectra of 50 μM peptides in trifluoroethanol (TFE) were recorded on a JASCO J-815 (Japan) automatic recording spectropolarimeter. The spectra were recorded at 25 °C using a quartz cell of 1 mm path length. The data obtained has been represented as mean residue ellipticities.

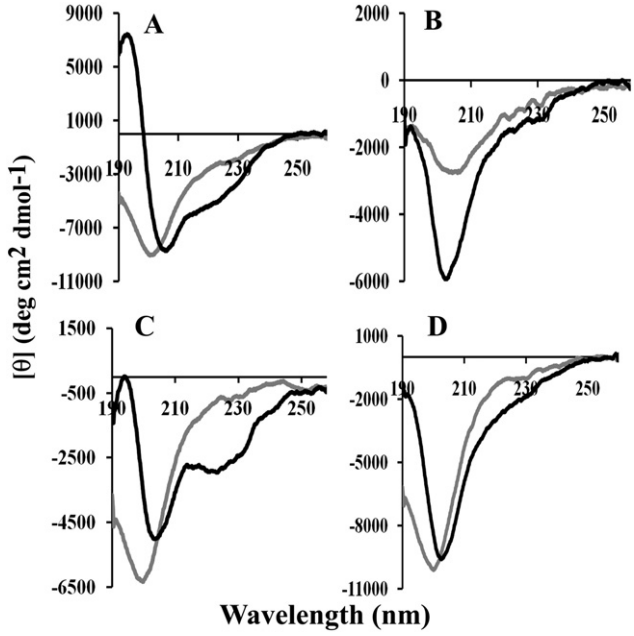

Fig. 2. CD spectra of acetylated and palmitoylated BNBD-2 analogs in 5 mM HEPES buffer pH 7.4 and containing 12 mM SDS. (A) PA1L, (B) PA1, (C) AC1L and (D) AC1. Key: gray line — buffer, dark line — 12 mM SDS.

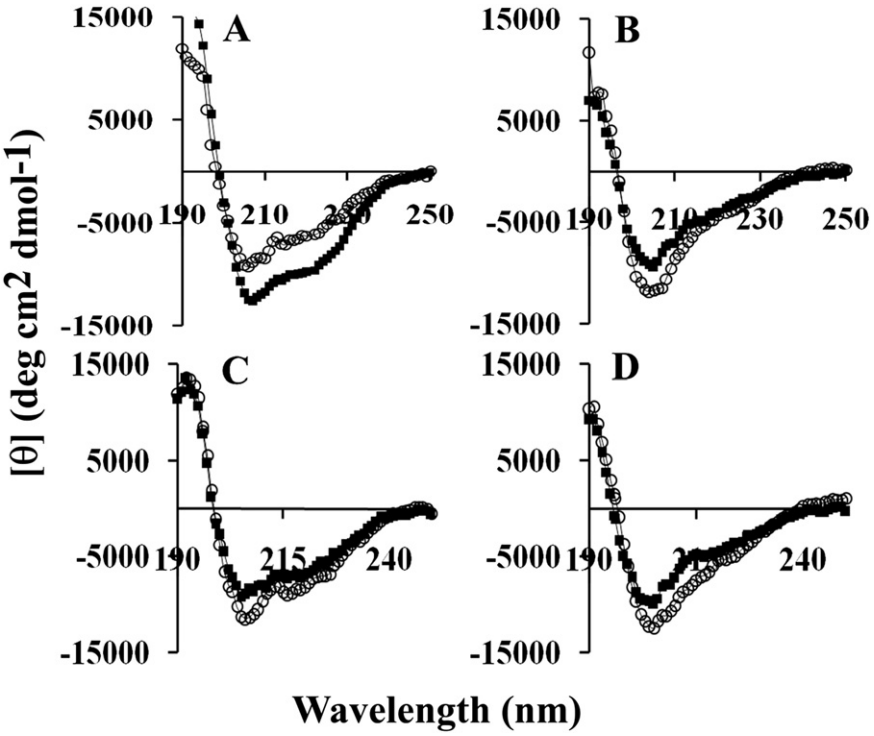

Fig. 3. CD spectra of palmitoylated and acetylated BNBD-2 analogs in TFE. Key: (A) PA1L and PA1 (B) PA4L and PA4 (C) AC1L and AC1 (D) AC4L and AC4. Key: (■) linear peptides, (O) cylic peptides.

2.6. Preparation of large unilamellar vesicles (LUVs)

The lipid vesicles were prepared as follows: 5 mM POPC lipids were dried under vacuum overnight. The lipid film thus obtained was hydrated with 1 ml of 5 mM HEPES buffer, pH 7.4 and the solution was subjected to vortex mixing. The multilamellar vesicles obtained were extruded 40 times through a Lipofast extruder apparatus (Avestin Inc., Ottawa, ON) equipped with a polycarbonate membrane filter (19 mm diameter, 100 nm pore diameter) to produce LUVs with vesicle size of 100 nm [46]. The size distribution of vesicles was confirmed by dynamic light scattering (DLS) measurements.

2.7. Calcein leakage assay

Large unilamellar lipid vesicles composed of POPC: Cholesterol (10:1) or POPC: POPG (1:1) entrapped with calcein (50 mM) were prepared by extrusion through polycarbonate membranes with a pore size of 100 nm on a Lipofast extruder apparatus (Avestin, Inc., Canada) [46]. Lipid film was hydrated with 5 mM HEPES buffer pH 7.4, 50 mM NaCl containing 50 mM calcein. After extrusion, untrapped calcein was removed by gel filtration on a Sephadex G-75 column. Leakage of dye from lipid vesicles was determined as follows: small aliquots of calcein-entrapped vesicles (75 μM) were added to 0.5 ml of 5 mM HEPES buffer pH 7.4, 150 mM NaCl containing peptide in a quartz cuvette. Fluorescence was measured in a Hitachi F-4500 Fluorescence spectrophotometer with excitation at 490 nm and emission at 520 nm. The excitation and emission band widths were 3 and 5 nm respectively. The maximum fluorescence intensity was determined by addition of Triton X-100 to a final concentration of 0.1%.

2.8. Inner-membrane permeabilization (IM)

Inner-membrane permeability of *E. coli* in the presence of the acylated peptides was assessed by using o-nitrophenyl-β-D-galactoside (ONPG), a substrate for cytoplasmic β-galactosidase enzyme [47]. *E. coli* strain (GJ 2455), which is constitutive for β-galactosidase synthesis (derivative of *E. coli* MG 1655 lac I^-, lac Z^+), was grown to mid log phase in LB broth. The cells were diluted to an OD_{600} of 0.05 with PB containing 20 mM ONPG. Aliquots of these cells were incubated with fixed concentration of peptide (30 μM) at 37 °C for 30 min. OD measurement made at 420 nm which reflects ONPG influx into the cells, was taken as an indicator of the permeability status of IM. Membrane permeabilization kinetics was also determined for a representative set of palmitoylated (PA1) and acetylated (AC1) peptides by following the kinetics of hydrolysis of ONPG to cytoplasmic galactosidases. Controls without peptides were taken as blanks. The experiments were repeated thrice with duplicate samples. Activity obtained from cells incubated with SDS (1% (w/v)) was taken as 100%.

2.9. Hemolytic assay

Human erythrocytes were isolated from heparinized human blood by centrifugation and washed thrice with phosphate-buffered saline (PBS, 10 mM phosphate buffer containing 0.15 M NaCl, pH 7.4) just before assays were performed. Aliquots of cell suspension (10^7/ml) in Eppendorf tubes were incubated at 37 °C with peptides in duplicates for 30 min with gentle mixing. The samples were centrifuged and the absorbance of the supernatant was measured at 540 nm. The lysis obtained with 1% Triton X-100 was considered as 100%. The percentage lysis was calculated. Human serum (20%) was included in the incubation buffer to determine the effect of serum on the activity. Hemolytic activity in the presence of POPC large unilamellar vesicles (LUVs) was also determined at varying lipid peptide ratios. The experiments were repeated thrice with duplicate samples. The percentage lysis was calculated as described above.

3. Results

The primary structures of peptides chosen for the study are shown in Table 1. It was earlier reported that the C-terminal segment of BNBD-2

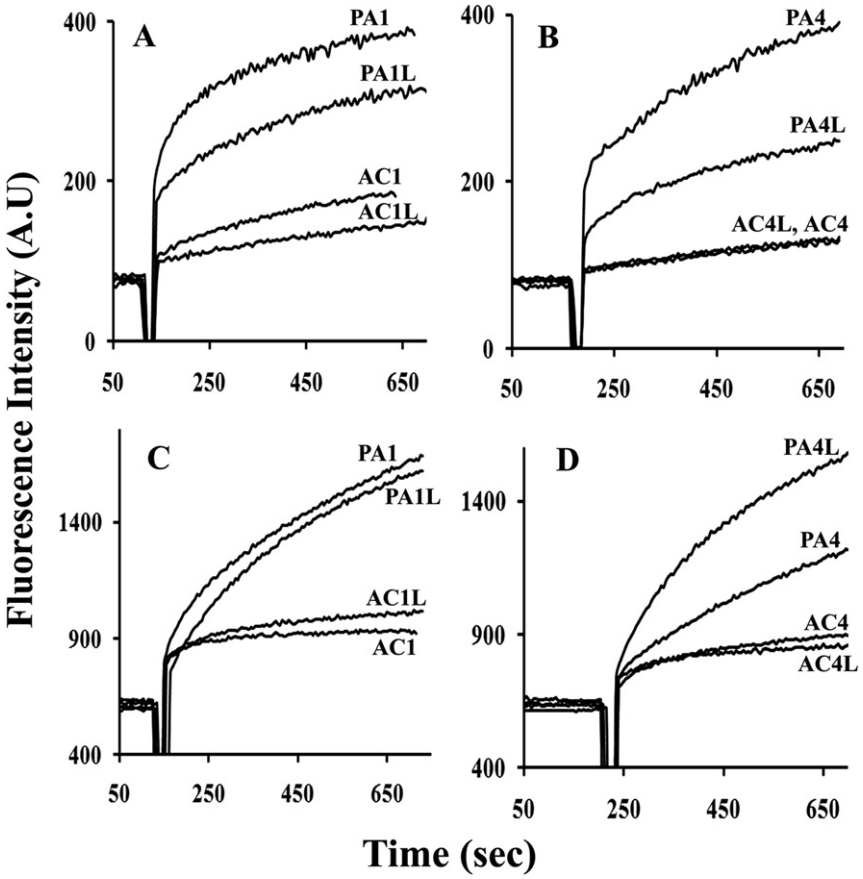

Fig. 4. Calcein release from lipid vesicles in the presence of acylated peptides. The acylated peptides (final concentration 3 μM) were added to LUVs with different phospholipid compositions encapsulated with calcein. (A) Calcein release induced by palmitoylated and acetylated peptides (PA1L, PA1, AC1L and AC1) from POPC:POPG (1:1) vesicles. (B) Calcein release induced by palmitoylated and acetylated peptides (PA4L, PA4, AC4L and AC4) from POPC:POPG (1:1) vesicles. Peptide/lipid ratios are 1:250. (C) Calcein release induced by palmitoylated and acetylated peptides (PA1L, PA1, AC1L and AC1) from POPC:cholesterol (10:1) vesicles. (D) Calcein release induced by palmitoylated and acetylated peptides (PA4L, PA4, AC4L and AC4) from POPC:cholesterol (10:1) vesicles. Peptide/lipid ratios are 1:25.

(PB1), its variant (PB4) in which G and P (14th and 15th residues) in the sequence PB1 were reversed to (PG) and prolines were replaced, with D-proline (^{D}P) exhibits antibacterial activity [14]. N-terminal palmitoylated and acetylated single disulfide bond containing peptides (PA1, PA4, AC1 and AC4) and their corresponding linear forms with (acm), side chain protecting group in cysteines (PA1L, PA4L, AC1L, and AC4L) were derived from PB1 and PB4. N-terminal acetylated peptides were synthesized to examine the effect of blocking the N-terminus. The synthetic peptides were analyzed on HPLC using reversed phase C18 column. The palmitoylated peptides (PA1L, PA1, PA4 and PA4L) eluted between 50 and 53 min and acetylated peptides (AC1, AC4, AC1L, and AC4L) between 31 and 33 min, indicated considerable increase in hydrophobicity for the palmitoylated peptides.

The antimicrobial activity of palmitoylated (PA1L, PA1, PA4L and PA4) and acetylated (AC1L, AC1, AC4 and AC4L) peptides examined by the method used to determine the antimicrobial activity of mammalian defensins [42–45] is summarized in Table 2. Both linear and single disulfide bond containing peptides show antimicrobial activity. The data indicate that N-terminal modified peptides did not show significant enhancement in antibacterial activity against *E. coli* and *P. aeruginosa* when compared to the unacylated BNBD-2 analogs (shown with *). A marginal improvement in antibacterial activity was observed for all the peptides except for PA1L against *S. aureus*. Slight increase in antifungal activity was observed for N-terminal acetylated and palmitoylated disulfide bond containing peptides. The data indicate that N-terminal modification by acetylation or palmitoylation does not result in increased antimicrobial potency.

The effect of 150 mM NaCl and 20% human serum on antibacterial activity against *E. coli* and *S. aureus* was also determined. The results show that palmitoylated peptides exhibited activity in the presence of NaCl whereas unacylated and acetylated BNBD-2 peptides did not show activity under similar conditions. In the presence of 20% serum, loss of activity of both palmitoylated and acetylated peptides was observed only against *S. aureus*.

We further investigated biophysical properties such as tendency to aggregate, structure in membrane-mimicking environment and ability to permeabilize model membranes to get insights into salt resistant antimicrobial activity of the palmitoylated peptides.

The linear palmitoylated (PA1L, PA4L) and single disulfide containing peptides (PA1, PA4) are cationic peptide segments with a hydrophobic fatty acyl chain at the end, similar to micelle forming amphiphatic peptides. ANS is a fluorescent probe that changes its intensity or emission maximum depending on the polarity of the environment [48]. The aggregation property of palmitoylated peptides (PA1L PA1, PA4L, PA4) was investigated by monitoring the changes in intensity of ANS fluorescence at 480 nm (Fig. 1 (A–D)) in the presence and absence of 150 mM NaCl. When the fluorescence intensity at 480 nm was plotted as a function of the peptide concentration, linear increase was observed for all of the peptides indicating the absence of micelle formation. Since there is an increase in ANS fluorescence, it is likely that the peptides aggregate, but not into distinctive micellar structures like SDS or CTAB. The acetylated peptides did not show any change in fluorescence intensity (data not shown).

The secondary structure of palmitoylated (PA1L, PA1) and acetylated (AC1L and AC1) peptides was examined by CD spectroscopy in

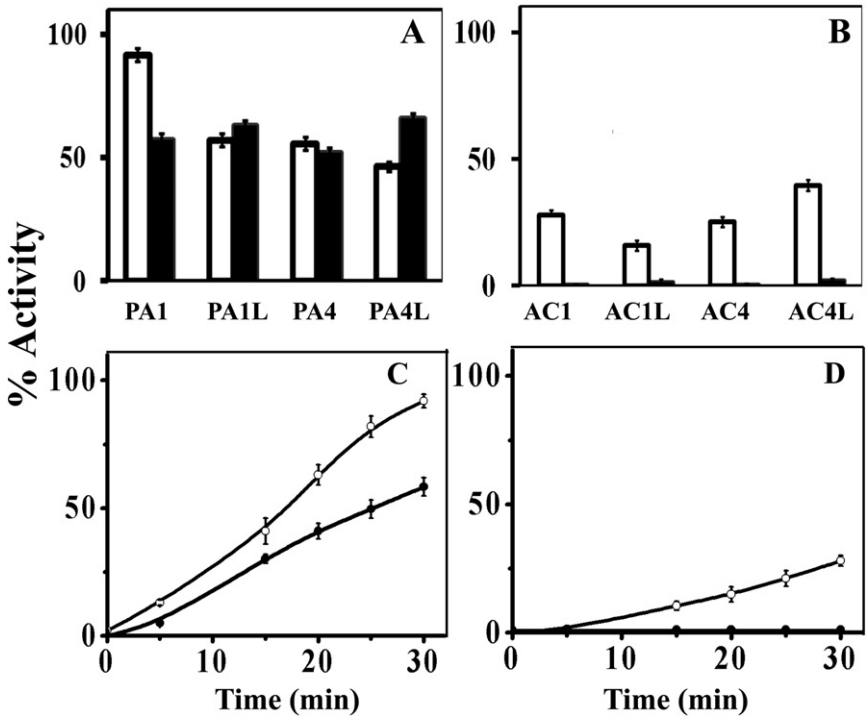

Fig. 5. Effect of palmitoylated and acetylated peptides on inner membrane permeability. Mid-log-phase *E. coli* GJ 2544 was incubated with 20 mM ONPG and the release of ONP due to hydrolysis ONPG by cytoplasmic β-galactosidase was monitored at 420 nm. Complete hydrolysis of ONPG with 1% SDS after 30 min was taken as 100%. Cells incubated in the absence of peptide were taken as the control. Inner membrane permeabilization measured at 30 min after the addition (30 μM) palmitoylated peptides (Fig. 5A) and acetylated peptides (Fig. 5B) in the presence and absence of 150 mM NaCl is shown. Key: open bars (− NaCl) and dark bars (+ NaCl). The kinetics of inner membrane permeabilization of a palmitoylated peptide PA1 shown in C and acetylated peptide AC1 in D. Open circles (− NaCl) and dark circles (+ NaCl). The error bars represent standard deviation.

10 mM phosphate pH 7.4 buffer, and in the presence of 12 mM SDS (shown in Fig. 2 (A–D)). SDS provides a membrane-like environment and has been used extensively as model membrane system [49]. In buffer, all the peptides spectra show a minimum ~195 nm and cross over below 195 nm indicating the absence of ordered conformation of these peptides (Fig. 2 (A–D gray lines)). PA1, AC1L and AC1 show a negative band at ~195 nm and a broad negative band at 200–220 nm in the presence of 12 mM SDS, which is above CMC of SDS indicating unordered conformation Fig. 2 (B–D dark lines). PA1L showed a negative band at 207 nm and shoulder at 220 nm which is typical of α-helical structure Fig. 2 (A, dark line).

The CD spectra of palmitoylated and acetylated peptides recorded in TFE are shown in Fig. 3 (A–D). In TFE, PA1L, PA1, AC1L and AC1 show negative minima at 205 and 225 nm with a crossover at 200 nm. The spectra are similar to peptides populating helical and β-hairpin conformation [50,51] and defensins with three disulfide bridges [52,53]. Introduction of ^{D}P and switching of GP to PG in PA4L, PA4, AC4L and AC4 appears to favor β-hairpin conformation as shown in Fig. 3B (PA4L, PA4) and Fig. 3D (AC4L, AC4). The results indicate that in a medium like TFE, no significant differences in the secondary structure were observed upon acetylation or palmitoylation of peptides.

The ability of acylated peptides to permeabilize model membranes was investigated by monitoring the release of calcein from lipid vesicles with varying lipid compositions. Lipid vesicles composed of POPC:POPG (1:1) and POPC:cholesterol (10:1) are often used extensively to investigate peptide–membrane interactions [54–57]. Calcein release in the presence of palmitoylated and acetylated peptides is shown in Fig. 4 (A–D). Release of calcein from POPC:POPG (1:1) is shown in Fig. 4 (A–B), and POPC:cholesterol (10:1) is shown in Fig. 4 (C–D). The data indicate that palmitoylated peptides were more effective in causing calcein release from POPC:POPG (1:1) or POPC:cholesterol (10:1) vesicles compared to acetylated peptides.

Inner-membrane permeabilization was determined by monitoring the cytoplasmic-β-galactosidase activity after 30 min in the presence of acylated peptides. Inner-membrane permeabilization of *E. coli* by palmitoylated (PA1L, PA1, PA4L and PA4) and acetylated (AC1L, AC1, AC4 and AC4L) peptides (30 μM) in the presence and absence of 150 mM NaCl is shown in Fig. 5 (A–B). It was observed that palmitoylated peptides (PA1L, PA1, PA4L and PA4) permeabilized *E. coli* inner-membrane more effectively in the presence and absence NaCl (shown in Fig. 5A) when compared to acetylated peptides (shown in Fig. 5B). The kinetics of inner membrane permeabilization of a representative set of palmitoylated (PA1) and acetylated peptide (AC1) are shown in Fig. 5 (C, D). The data showed different permeabilization kinetics for PA1 and AC1 (Fig. 5C, D). Inner-membrane permeabilization was more rapid in the presence of PA1 (Fig. 5C), that is reflected in the antimicrobial activity of PA1, both in the presence and absence of NaCl.

Hemolytic activity of the palmitoylated (PA1L, PA1, PA4L and PA4) and acetylated (AC1L, AC1, AC4 and AC4L) peptides is shown in Fig. 6 (A–E). The N-terminal modified analogs (both acetylated and palmitoylated) showed hemolytic activity. N-terminal palmitoylated peptides showed considerably more hemolytic activity when compared to acetylated peptides. Significant decrease in hemolytic activity was observed in the presence of 20% human serum as shown in Fig. 6 (C–D). Hemolytic activity was also considerably reduced in the presence of POPC vesicles with increasing peptide/lipid molar ratios (Fig. 6E). However, antibacterial activity was not affected in the presence of POPC vesicles (data not shown). These results indicate that N-terminal blocking of the peptide with palmitoylation increases hemolytic activity. Significant decrease in hemolytic activity without affecting antibacterial activity in the presence of lipid vesicles indicates selectivity in activity can be obtained by liposomal formation.

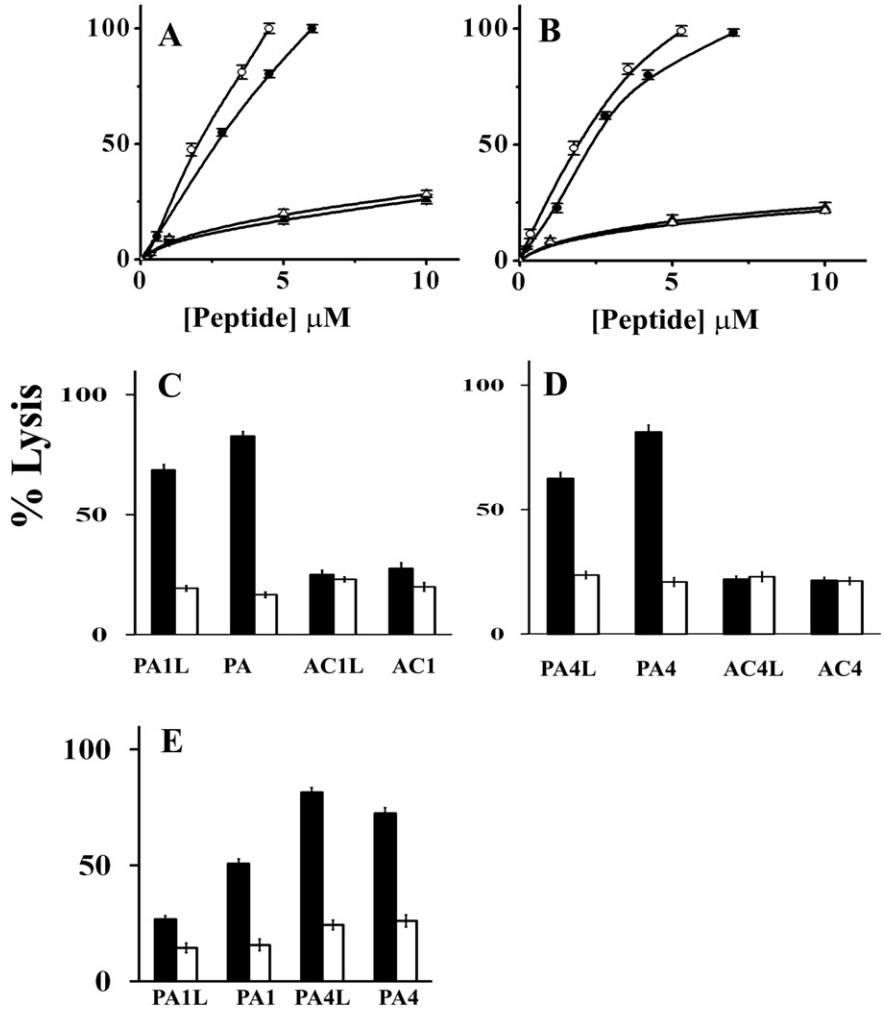

Fig. 6. Hemolytic activity of linear and cyclic variant of palmitoylated and acetylated BNBD-2 analogs. (A, B) Hemolysis of palmitoylated and acetylated peptides as a function of peptide concentration (A) PA1L, PA1, AC1L and AC1 (B) PA4L, PA4, AC4L and AC4. Key: (O) PA1 or PA4; (●) PA1L or PA4L; (Δ) AC1 or AC4; (▲) AC1L or AC4L; (C, D) effect of 20% human serum on hemolytic activity of palmitoylated and acetylated BNBD-2 analogs at a fixed concentration of peptide (3 μM). Key: dark bars (− serum) and open bars (+ serum). (E) Protection of hemolytic activity of palmitoylated peptides (PA1L, PA1, PA4L and PA4) in the presence of POPC vesicles at different molar ratios of peptide to lipid. Key: dark bars (1:20), open bars (1:100). The error bars represent the standard deviation.

4. Discussion

α- and β-defensins show broad spectrum antimicrobial activity [1–4]. However, physiological concentrations of salt attenuate their antimicrobial activity considerably [7,11,14,25]. Several approaches have been used to engineer defensin analogs to render their antimicrobial activity salt-insensitive. For example, salt sensitive NP1 (α-defensin) analogs when they were end to end circularized, i.e. β-tile peptides with clustered cationic residues, retained activity in the presence of NaCl against *E. coli* and *Salmonella typhimurium* [58]. The chimeric human beta defensins HBD-1/HBD-3 [24] and HBD-2/HBD-3 [25] analogs identified different domains or segments in HBD-1–3 that are critical for specific antibacterial activity and salt sensitivity [24,25]. These strategies involved de novo chemical synthesis. Truncated analogs of α- and β-defensins exhibit antimicrobial activity indicating that the entire sequence or the structural features observed in native defensins is not essential for activity. However, many of these analogs are inactive in the presence of physiological concentration of salts such as Na^+ and divalent cations. A relatively simple strategy to generate peptides that retain antimicrobial activity in the presence of salt was explored. While the C-terminal segment of BNBD-2 is rich in cationic amino acids, particularly arginine residues, it is devoid of hydrophobic amino acids. The hydrophobicity of the peptide was increased by covalently adding the fatty acid i.e. palmitic acid at the N-terminus. The corresponding acetylated peptides were also generated to study the effect of N-terminal capping of the peptide.

Earlier studies on unacylated BNBD-2 analogs, which have free amine group at the N-terminus have indicated that the loss of antibacterial activity in the presence of salt could arise due to inability of the peptides to associate with the membranes [14]. Studies on fatty acylated peptides showed that the presence of a lipophilic moiety can provide hydrophobicity that can modulate antibacterial activity but does not always result in enhanced activity [27,34,59]. Increase in leishmanicidal activity was observed for N-terminal fatty acylated cecropin–melittin hybrid peptide [27]. Similarly, improved antibacterial activity was observed for a 9-residue peptide derived from lactoferrin B [60]. Acylation with long chain fatty acids resulted in loss of activity against *S. aureus* and *E. coli* in dermaseptin derivatives [34]. In maganin analogs MS1-78 and LL37, the antibacterial activity was inhibited considerably whereas enhancement in hemolytic activity was observed [34].

The results presented in this paper indicate that while fatty acylation does not enhance the antimicrobial potency, the activity was not attenuated by high concentrations of NaCl. N-terminal fatty acylation appears

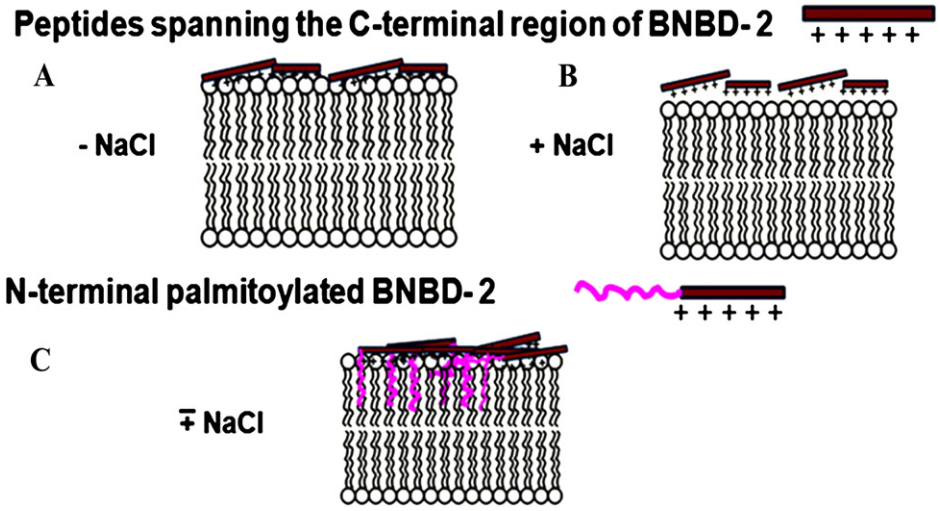

Fig. 7. A model for association of palmitoylated peptides derived from cationic C-terminal segment of BNBD-2 with the lipid bilayer. Binding of the unacylated BNBD-2 analogs in the absence (A), and in the presence (B) of NaCl is shown. Binding of palmitoylated BNBD-2 analogs, where palmitoylated chain inserted into the bilayer in the presence and absence of NaCl (C). Circles represent lipid head groups. Thin zig-zag lines depict acyl chains of membrane phospholipids.

to favor greater interaction of the peptide chain with negatively charged head-group region of the bacterial membrane which is not affected in the presence of NaCl. The presence of a hydrophobic N-terminus and cationic peptide segment did not result in micelle formation in the range of antimicrobial and hemolytic activity. The greater membrane disruptive ability of palmitoylated peptides correlates with their antibacterial activity in the presence of NaCl as well as with their hemolytic activity. The disulfide bridged fatty acylated peptides did not adopt folded conformation in buffer or even in SDS. Only linear peptide particularly PA1L showed tendency to form helical structure in SDS, but this does not result in enhanced antibacterial activity. Clearly, the hemolytic activity is also caused by membrane destabilization rather than pore formation by ordered aggregates of peptides as even the linear peptides that tend to adopt helical conformation in SDS micelles are less hemolytic as compared to the disulfide bridged peptides. The palmitoylated and acetylated peptides also showed similar structures in the presence of TFE which indicate that a definite secondary structure may not be crucial for antimicrobial activity of palmitoylated peptides in the presence of salt. A schematic representation of the interaction of fatty acylated peptides with model membranes is shown in Fig. 7. When peptide molecules encounter lipids, the fatty acyl chain inserts into the lipid bilayer providing an anchor and facilitating greater contact of the positively charged residues with the negatively charged membrane surface which is not disrupted by high NaCl concentrations. Subsequently membrane destabilization occurs, which results in the membrane permeabilization. Since the peptides are not amphipathic, it is unlikely that membrane permeabilization is by toroidal pore formation [61]. The model membrane binding ability of these peptides was exploited to overcome undesirable hemolytic activity. Considerable reduction in hemolytic activity without reduction in antimicrobial activity was observed when palmitoylated peptides were associated with zwitterionic lipid vesicles.

Acylated peptides showed activity in the presence of serum and inhibition of activity in the presence of heat inactivated serum against *E. coli* (data not shown). This result is similar to that obtained with polymyxin B that showed bactericidal activity against Gram-negative bacteria in the presence of human serum [62]. Palmitoylation does not result in interaction with serum components as activity against *E. coli* is not diminished, it is possible that serum components bind to the cell surface of *S. aureus* and prevent binding of peptides. It has been hypothesized that the loss of activity in the presence of heat inactivated serum could be due to the blocking or inhibitory factors generated during heat inactivation that influence the activity of peptides [63].

Thus, the study shows that truncated defensin analogs modified with fatty acids and without having the structural features of parent defensins can exhibit antibacterial activity. The results indicate that a facile modification of defensin analogs can result in enhanced salt tolerance and complex restructuring of the peptide is not required. A design that permits strong interaction of cationic segments with membrane surface results in antimicrobial activity. By simple engineering of defensins it should be possible to get molecules that could be attractive candidates for therapeutic development.

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References

[1] T. Ganz, Defensins: antimicrobial peptides of vertebrates, C. R. Biol. 327 (2004) 539–549.
[2] R.I. Lehrer, T. Ganz, Defensins of vertebrate animals, Curr. Opin. Immunol. 14 (2002) 96–102.
[3] J.J. Oppenheim, A. Biragyn, L.W. Kwak, D. Yang, Roles of antimicrobial peptides such as defensins in innate and adaptive immunity, Ann. Rheum. Dis. 62 (2003) 17–21.
[4] M. Pazgier, D.M. Hoover, D. Yang, W. Lu, J. Lubkowski, Human beta-defensins, Cell. Mol. Life Sci. 63 (2006) 1294–1313.
[5] J.J. Schneider, A. Unholzer, M. Schaller, M. Schafer-Korting, H.C. Korting, Human defensins, J. Mol. Med. 83 (2005) 587–595.
[6] M.E. Selsted, A.J. Ouellette, Mammalian defensins in the antimicrobial immune response, Nat. Immunol. 6 (2005) 551–557.
[7] D.M. Hoover, O. Chertov, J. Lubkowski, The structure of human beta-defensin-1: new insights into structural properties of beta-defensins, J. Biol. Chem. 276 (2001) 39021–39026.
[8] M.V. Sawai, H.P. Jia, L. Liu, V. Aseyev, J.M. Wiencek, P.B. McCray Jr., T. Ganz, W.R. Kearney, B.F. Tack, The NMR structure of human beta-defensin-2 reveals a novel alpha-helical segment, Biochemistry 40 (2001) 3810–3816.
[9] J. Harder, J. Bartels, E. Christophers, J.M. Schroder, Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic, J. Biol. Chem. 276 (2001) 5707–5713.
[10] R. Bals, X. Wang, Z. Wu, T. Freeman, V. Bafna, M. Zasloff, J.M. Wilson, Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung, J. Clin. Invest. 102 (1998) 874–880.
[11] M.J. Goldman, G.M. Anderson, E.D. Stolzenberg, U.P. Kari, M. Zasloff, J.M. Wilson, Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis, Cell 88 (1997) 553–560.

[12] J. Harder, J. Bartels, E. Christophers, J.M. Schroder, A peptide antibiotic from human skin, Nature 387 (1997) 861.
[13] D.M. Hoover, Z. Wu, K. Tucker, W. Lu, J. Lubkowski, Antimicrobial characterization of human beta-defensin 3 derivatives, Antimicrob. Agents Chemother. 47 (2003) 2804–2809.
[14] V. Krishnakumari, A. Sharadadevi, S. Singh, R. Nagaraj, Single disulfide and linear analogues corresponding to the carboxy-terminal segment of bovine beta-defensin-2: effects of introducing the beta-hairpin nucleating sequence d-pro-gly on antibacterial activity and Biophysical properties, Biochemistry 42 (2003) 9307–9315.
[15] V. Krishnakumari, S. Singh, R. Nagaraj, Antibacterial activities of synthetic peptides corresponding to the carboxy-terminal region of human beta-defensins 1–3, Peptides 27 (2006) 2607–2613.
[16] E. Kluver, K. Adermann, A. Schulz, Synthesis and structure–activity relationship of beta-defensins, multi-functional peptides of the immune system, J. Pept. Sci. 12 (2006) 243–257.
[17] M. Mandal, R. Nagaraj, Antibacterial activities and conformations of synthetic alpha-defensin HNP-1 and analogs with one, two and three disulfide bridges, J. Pept. Res. 59 (2002) 95–104.
[18] M. Pazgier, X. Li, W. Lu, J. Lubkowski, Human defensins: synthesis and structural properties, Curr. Pharm. Des. 13 (2007) 3096–3118.
[19] H.G. Sahl, U. Pag, S. Bonness, S. Wagner, N. Antcheva, A. Tossi, Mammalian defensins: structures and mechanism of antibiotic activity, J. Leukoc. Biol. 77 (2005) 466–475.
[20] Z. Wu, D.M. Hoover, D. Yang, C. Boulegue, F. Santamaria, J.J. Oppenheim, J. Lubkowski, W. Lu, Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 8880–8885.
[21] S. Liu, L. Zhou, J. Li, A. Suresh, C. Verma, Y.H. Foo, E.P. Yap, D.T. Tan, R.W. Beuerman, Linear analogues of human beta-defensin 3: concepts for design of antimicrobial peptides with reduced cytotoxicity to mammalian cells, Chembiochem 9 (2008) 964–973.
[22] A.J. Wommack, S.A. Robson, Y.A. Wanniarachchi, A. Wan, C.J. Turner, G. Wagner, E.M. Nolan, NMR solution structure and condition-dependent oligomerization of the antimicrobial peptide human defensin 5, Biochemistry 51 (2012) 9624–9637.
[23] Y.A. Wanniarachchi, P. Kaczmarek, A. Wan, E.M. Nolan, Human defensin 5 disulfide array mutants: disulfide bond deletion attenuates antibacterial activity against *Staphylococcus aureus*, Biochemistry 50 (2011) 8005–8017.
[24] O. Scudiero, S. Galdiero, M. Cantisani, R. Di Noto, M. Vitiello, M. Galdiero, G. Naclerio, J.J. Cassiman, C. Pedone, G. Castaldo, F. Salvatore, Novel synthetic, salt-resistant analogs of human beta-defensins 1 and 3 endowed with enhanced antimicrobial activity, Antimicrob. Agents Chemother. 54 (2010) 2312–2322.
[25] S. Jung, J. Mysliwy, B. Spudy, I. Lorenzen, K. Reiss, C. Gelhaus, R. Podschun, M. Leippe, J. Grotzinger, Human beta-defensin 2 and beta-defensin 3 chimeric peptides reveal the structural basis of the pathogen specificity of their parent molecules, Antimicrob. Agents Chemother. 55 (2011) 954–960.
[26] J. Greaves, G.R. Prescott, O.A. Gorleku, L.H. Chamberlain, The fat controller: roles of palmitoylation in intracellular protein trafficking and targeting to membrane microdomains, Mol. Membr. Biol. 26 (2008) 67–79.
[27] C. Chicharro, C. Granata, R. Lozano, D. Andreu, L. Rivas, N-terminal fatty acid substitution increases the leishmanicidal activity of CA(1–7)M(2–9), a cecropin–melittin hybrid peptide, Antimicrob. Agents Chemother. 45 (2001) 2441–2449.
[28] D. Avrahami, Y. Shai, A new group of antifungal and antibacterial lipopeptides derived from non-membrane active peptides conjugated to palmitic acid, J. Biol. Chem. 279 (2004) 12277–12285.
[29] T. Etzerodt, J.R. Henriksen, P. Rasmussen, M.H. Clausen, T.L. Andresen, Selective acylation enhances membrane charge sensitivity of the antimicrobial peptide mastoparan-x, Biophys. J. 100 (2011) 399–409.
[30] Z. Li, P. Yuan, M. Xing, Z. He, C. Dong, Y. Cao, Q. Liu, Fatty acid conjugation enhances the activities of antimicrobial peptides, Recent Pat. Food Nutr. Agric. 5 (2013) 52–56.
[31] P. Mak, J. Pohl, A. Dubin, M.S. Reed, S.E. Bowers, M.T. Fallon, W.M. Shafer, The increased bactericidal activity of a fatty acid-modified synthetic antimicrobial peptide of human cathepsin G correlates with its enhanced capacity to interact with model membranes, Int. J. Antimicrob. Agents 21 (2003) 13–19.
[32] A. Makovitzki, Y. Shai, pH-dependent antifungal lipopeptides and their plausible mode of action, Biochemistry 44 (2005) 9775–9784.
[33] M.L. Mangoni, Y. Shai, Short native antimicrobial peptides and engineered ultrashort lipopeptides: similarities and differences in cell specificities and modes of action, Cell. Mol. Life Sci. 68 (2011) 2267–2280.
[34] I.S. Radzishevsky, S. Rotem, F. Zaknoon, L. Gaidukov, A. Dagan, A. Mor, Effects of acyl versus aminoacyl conjugation on the properties of antimicrobial peptides, Antimicrob. Agents Chemother. 49 (2005) 2412–2420.
[35] I.S. Radzishevsky, S. Rotem, D. Bourdetsky, S. Navon-Venezia, Y. Carmeli, A. Mor, Improved antimicrobial peptides based on acyl-lysine oligomers, Nat. Biotechnol. 25 (2007) 657–659.
[36] R.W. Scott, W.F. DeGrado, G.N. Tew, De novo designed synthetic mimics of antimicrobial peptides, Curr. Opin. Biotechnol. 19 (2008) 620–627.
[37] H. Tsubery, I. Ofek, S. Cohen, M. Fridkin, N-terminal modifications of polymyxin B nonapeptide and their effect on antibacterial activity, Peptides 22 (2001) 1675–1681.
[38] E. Atherton, R.C. Sheppard, Solid Phase Peptide Synthesis: A Practical Approach, Oxford University Press, 1989.
[39] A. Harishchandran, B. Pallavi, R. Nagaraj, A synthetic strategy for on-resin amino acid specific multiple fatty acid acylation of peptides, Protein Pept. Lett. 9 (2002) 411–417.
[40] D.F. Veber, J.D. Milkowski, S.L. Varga, R.G. Denkewalter, R. Hirschmann, Acetamidomethyl. A novel thiol protecting group for cysteine, J. Am. Chem. Soc. 94 (1972) 5456–5461.
[41] J.P. Tam, C.R. Wu, W. Liu, J.W. Zhang, Disulfide bond formation in peptides by dimethyl sulfoxide. Scope and applications, J. Am. Chem. Soc. 113 (1991) 6657–6662.
[42] R.I. Lehrer, M. Rosenman, S.S. Harwig, R. Jackson, P. Eisenhauer, Ultrasensitive assays for endogenous antimicrobial polypeptides, J. Immunol. Methods 137 (1991) 167–173.
[43] B. Ericksen, Z. Wu, W. Lu, R.I. Lehrer, Antibacterial activity and specificity of the six human α-defensins, Antimicrob. Agents Chemother. 49 (2005) 269–275.
[44] S. Vylkova, N. Nayyar, W. Li, M. Edgerton, Human β-defensins kill *Candida albicans* in an energy-dependent and salt-sensitive manner without causing membrane disruption, Antimicrob. Agents Chemother. 51 (2007) 154–161.
[45] V. Krishnakumari, N. Rangaraj, R. Nagaraj, Antifungal activities of human beta-defensins HBD-1 to HBD-3 and their C-terminal analogs Phd1 to Phd3, Antimicrob. Agents Chemother. 53 (2009) 256–260.
[46] R.C. MacDonald, R.I. MacDonald, B.P. Menco, K. Takeshita, N.K. Subbarao, L.R. Hu, Small-volume extrusion apparatus for preparation of large, unilamellar vesicles, Biochim. Biophys. Acta 1061 (1991) 297–303.
[47] R.I. Lehrer, A. Barton, K.A. Daher, S.S. Harwig, T. Ganz, M.E. Selsted, Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity, J. Clin. Invest. 84 (1989) 553–561.
[48] H.I. Park, S. Lee, A. Ullah, Q. Cao, Q.X.A. Sang, Effects of detergents on catalytic activity of human endometase/matrilysin 2, a putative cancer biomarker, Anal. Biochem. 396 (2010) 262–268.
[49] S.E. Blondelle, K. Lohner, M.I. Aguilar, Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: determination and biological specificity, Biochim. Biophys. Acta 1462 (1999) 89–108.
[50] F.J. Blanco, M.A. Jimenez, A. Pineda, M. Rico, J. Santoro, J.L. Nieto, NMR solution structure of the isolated N-terminal fragment of protein-G B1 domain. Evidence of trifluoroethanol induced native-like beta-hairpin formation, Biochemistry 33 (1994) 6004–6014.
[51] A.R. Viguera, M.A. Jiménez, M. Rico, L. Serrano, Conformational analysis of peptides corresponding to β-hairpins and α-sheet that represent the entire sequence of the -spectrin SH3 domain, J. Mol. Biol. 255 (1996) 507–521.
[52] N.F. Dawson, D.J. Craik, A.M. Mcmanus, S.G. Dashper, E.C. Reynolds, G.W. Tregear, O.J. Otvos, J.D. Wade, Chemical synthesis, characterization and activity of RK-1, a novel α-defensin-related peptide, J. Pept. Sci. 6 (2000) 19–25.
[53] T.M. Weiss, L. Yang, L. Ding, A.J. Waring, R.I,. Lehrer, H.W. Huang, Two states of cyclic antimicrobial peptide RTD-1 in lipid bilayers, Biochemistry 41 (2002) 10070–10076.
[54] T. Wieprecht, M. Dathe, M. Beyermann, E. Krause, W.L. Maloy, D.L. MacDonald, M. Bienert, Peptide hydrophobicity controls the activity and selectivity of magainin 2 amide in interaction with membranes, Biochemistry 36 (1997) 6124–6132.
[55] A.J. Rezansoff, H.N. Hunter, W. Jing, I.Y. Park, S.C. Kim, H.J. Vogel, Interactions of the antimicrobial peptide Ac-FRWWHR-NH2 with model membrane systems and bacterial cells, J. Pept. Res. 65 (2005) 491–501.
[56] J.T. Cheng, J.D. Hale, M. Elliot, R.E. Hancock, S.K. Straus, Effect of membrane composition on antimicrobial peptides aurein 2.2 and 2.3 from Australian southern bell frogs, Biophys. J. 96 (2009) 552–565.
[57] A.J. Mason, W. Moussaoui, T. Abdelrahman, A. Boukhari, P. Bertani, A. Marquette, B. Bechinger, Structural determinants of antimicrobial and antiplasmodial activity and selectivity in histidine-rich amphipathic cationic peptides, J. Biol. Chem. 284 (2009) 119–133.
[58] Q. Yu, R.I. Lehrer, J.P. Tam, Engineered salt-insensitive alpha-defensins with end-to-end circularized structures, J. Biol. Chem. 275 (2000) 3943–3949.
[59] D. Avrahami, Y. Shai, Conjugation of a magainin analogue with lipophilic acids controls hydrophobicity, solution assembly, and cell selectivity, Biochemistry 41 (2002) 2254–2263.
[60] H. Wakabayashi, H. Matsumoto, K. Hashimoto, S. Teraguchi, M. Takase, H. Hayasawa, N-acylated and D enantiomer derivatives of a nonamer core peptide of lactoferricin B showing improved antimicrobial activity, Antimicrob. Agents Chemother. 43 (1999) 1267–1269.
[61] K. Matsuzaki, Magainins as paradigm for the mode of action of pore forming polypeptides, Biochim. Biophys. Acta 1376 (1998) 391–400.
[62] P. Viljanen, H. Kayhty, M. Vaara, T. Vaara, Susceptibility of Gram-negative bacteria to the synergistic bactericidal action of serum and polymyxin B nonapeptide, Can. J. Microbiol. 32 (1986) 66–69.
[63] M.R. Yeaman, K.D. Gank, A.S. Bayer, E.P. Brass, Synthetic peptides that exert antimicrobial activities in whole blood and blood-derived matrices, Antimicrob. Agents Chemother. 46 (2002) 3883–3891.