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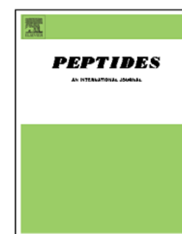

Antibacterial activities of synthetic peptides corresponding to the carboxy-terminal region of human β-defensins 1–3

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ABSTRACT

The antibacterial activities of synthetic human β-defensin analogs, constrained by a single disulfide bridge and in the reduced form, have been investigated. The peptides span the carboxy-terminal region of human β-defensins (HBD-1–3), which have a majority of cationic residues present in the native defensins. The disulfide constrained peptides exhibited activity against *Escherichia coli* and *Staphylococcus aureus* whereas the reduced forms were active only against *E. coli*. The antibacterial activities were attenuated in the presence of increasing concentrations of NaCl and divalent cations such as Ca^{2+} and Mg^{2+}. The site of action was the bacterial membrane. Peptides spanning the carboxy-terminal region of human β-defensins could be of help in understanding facets of antimicrobial activity of β-defensins such as salt sensitivity and mechanisms of bacterial membrane damage.

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1. Introduction

The isolation, characterization, tissue distribution, regulation and evaluation of antimicrobial activities of four human β-defensins (HBD-1–4) have been investigated extensively [6,17,22,25,32,40]. Computational analysis indicates that several genes code for human β-defensins in addition to HBD-1–4 [31]. However, the peptides corresponding to these sequences have not been isolated and characterized in detail. The disulfide connectivities in human β-defensins are similar to β-defensins from other mammalian sources [6,17,22,25,32,40]. HBD-1 and 2 have three β-strands in the carboxy-terminal region with a short stretch of helix in the amino-terminal region. These features are present both in the solid state and in solution [3,13,14,26]. The solution structure of HBD-3 suggests the presence of a similar structural motif [3,27].

Despite similar structures, the sequences of HBD-1–3 are different, especially with respect to the number of charged residues. Their antibacterial spectra also vary. HBD-1 and 2 are active predominantly against Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* [8–11,28,29,33,37,42]. They exhibit low activities against *Staphylococcus aureus*. These microbicidal activities are inhibited at high concentrations of NaCl. HBD-3 exhibits activity against Gram-negative and Gram-positive bacteria, which is not inhibited in the presence of physiological concentrations of NaCl [11]. Also, the order of disulfide pairing or the number of disulfide bonds are not important for exhibiting antibacterial activity in HBD-3 [15,18,39] as also observed in the α-defensin HNP-1 and bovine β-defensins BNBD-12 and BNBD-2 [20,23,24].

With a view to explore whether it is possible to generate active analogs of human β-defensins composed of fewer

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Peptide	Primary Sequence	Net Charge
HBD-1	DHYNC^{1}VSSGGQC^{2}LYSAC^{3}PIFTKIQGTC^{4}YRGKAKC^{5}C^{6}K	+4
Phd1	AC^{3}PIFTKIQGT--- YRGKAK---C^{6}K	+5
HBD-2	GIGDPVTC^{1}LKSGAIC^{2}HPVFC^{3}PRRYKQIGTC^{4}GLPGTKC^{5}C^{6}KKP	+6
Phd2	FC^3 PRRYKQIGT---GLPGTK---C^{6}K	+5
HBD-3	GIINTLQKYYC^{1}RVRGGRC^{2}AVLSC^{3}LPKEEQIGKC^{4}STRGRKC^{5}C^{6}RRKK	+11
Phd3	SC^{3}LPKEEQIGK---STRGRK---C^{6}RRKK	+7

α β$_1$ β$_2$ β$_3$

Fig. 1 – Primary structures of HBD-1–3 and analogs. Disulfide linkages are shown by horizontal lines. The regions forming helix (α) and β-strands (β$_{1-3}$) in HBD-1–3 are below in the sequences. The net positive charge is at pH 7.4. Gaps in Phd1–3 represent deleted cysteines. The reduced forms of Phd1–3 are denoted as Phdr 1–3 in the text.

residues and disulfide bridges as compared to the parent sequences, we have explored the antibacterial activities of synthetic peptides corresponding to the carboxy-terminal region of HBD-1–3. The sequences of full-length HBDs along with disulfide connectivities and the analogs investigated in the present study are shown in Fig. 1.

2. Materials and methods

2.1. *Materials*

4-(Hydroxymethyl) phenoxy acetamidomethyl resin (HMPA) and 9-fluorenylmethoxycarbonyl (F-moc) amino acids were obtained from Applied Biosystems (Foster City, CA) and Novabiochem AG (Switzerland), respectively. *N*-hydroxybenzotriazole hydrate (HOBT) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Advanced Chemtech (Louisville, KY). Reagents for deprotection of peptides were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. *Peptide synthesis*

Peptides were synthesized by solid-phase methods manually, using HMPA resin employing Fmoc chemistry [2]. Peptides were cleaved from the resin using trifluoroacetic acid containing thioanisole, meta-cresol and ethanedithiol (10:1:1:0.5, v/v). Formation of disulfide bonds was accomplished by air oxidation in 20% (v/v) aqueous dimethyl sulfoxide [34] at a concentration of 0.5 mg/ml for 24 h at room temperature. Peptides were purified by HPLC on a reversed phase C-18 (Hi-pore reversed phase column 4.6 mm × 250 mm) column using gradients of solvents: A; 0.1% (v/v) TFA in H_2O, B; 0.1% (v/v) TFA in CH_3CN. Purified peptides were characterized by Matrix-assisted laser desorption ionization time of flight mass spectrometry on a ABI Voyager DE STR MALDI-TOF mass spectrometer (Perseptive Biosystems) in the Proteomics Facility of CCMB using recrystallized α–cyano-4-hydroxycinnamic acid as matrix.

2.3. *Antibacterial activity*

Bacterial strains used were *E. coli* W 160-37 [38] and *S. aureus* (NCTC 8530). The antibacterial activity of the peptides was examined in sterile 96 well plates at a final volume of 100 μl as follows: Bacteria were grown in nutrient broth (Bacto Difco nutrient broth) to mid-log phase and diluted to 10^6 colony forming units (cfu)/ml in 10 mM sodium phosphate buffer (pH 7.4). Bacteria were incubated with different concentration of peptides for 2 h at 37 °C and suitably diluted aliquots were plated on nutrient agar plates. Dithiothreitol (DTT) (10 mM) was included in the buffer when activity of the reduced peptides were determined. After the plates were incubated at 37 °C for 18 h, colonies formed were counted. The concentration of the peptides at which no viable colonies were formed was taken as lethal concentration (LC). The LC determined is average of three independent experiments done in duplicate. To determine the effect of salt on antibacterial activity,

Table 1 – The antibacterial activity of human β-defensin analogs

Peptide	Lethal concentration; LC (μM)[a]	
	E. coli	*S. aureus*
Phd1	15	20
Phd2	18	23
Phd3	17	17

[a] Variations in LC between independent experiments were ±1 μM.

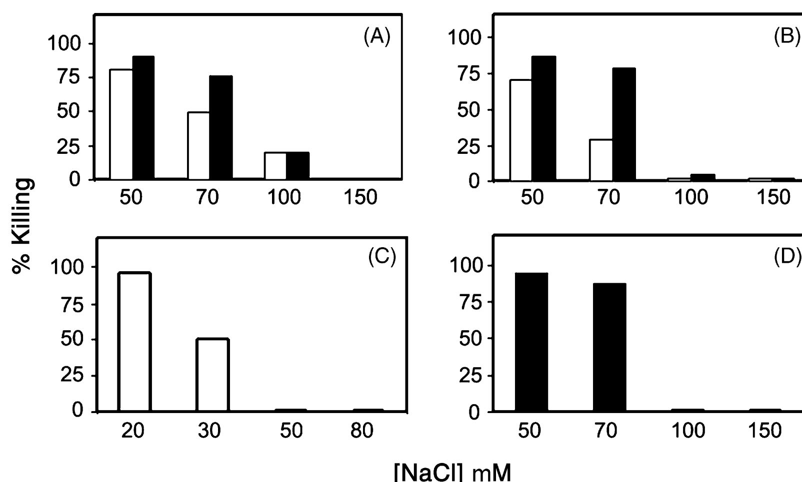

Fig. 2 – Salt dependence of antibacterial activity of HBD analogs Phd1–3. (A) Phd1, (B) Phd2, (C) and (D) Phd3. Open bars represent *E. coli* and dark bars represent *S. aureus*. Bacteria were incubated with Phd1–3 at their LC in 10 mM sodium phosphate buffer (pH 7.4) containing different concentrations of NaCl for 2 h at 37 °C and suitably diluted aliquots were plated on nutrient agar plates, which were incubated at 37 °C for 18 h. Colonies formed were counted and percentage bacteria killed was determined.

different concentrations of NaCl was included in the incubation buffer at their LC. Different concentrations of divalent cations were included in the buffer to determine their effect on activity at lethal concentration (LC) of the peptides.

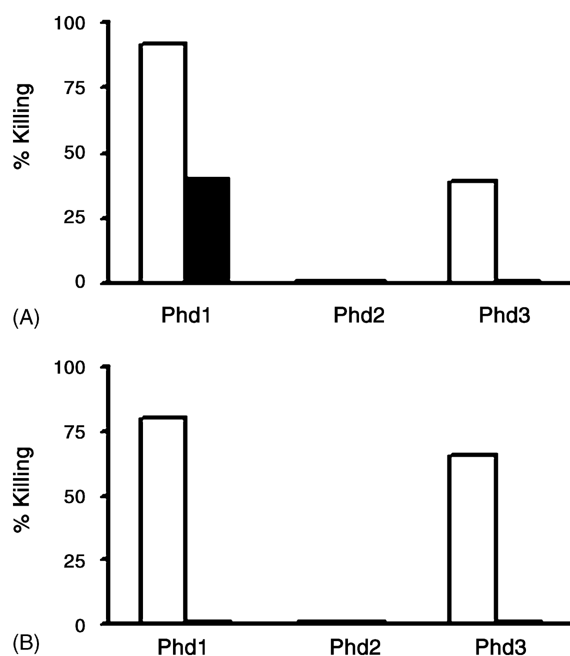

Fig. 3 – Effect of divalent cations on the antibacterial activity of HBD analogs Phd1–3 against *E. coli*. (A) $MgSO_4$ and (B) $CaCl_2$. Open and dark bars represent 0.25 and 2 mM salt concentrations, respectively. Bacteria were incubated with Phd1–3 at their LC in 10 mM sodium phosphate buffer (pH 7.4) containing different concentrations of $MgSO_4$ or $CaCl_2$ for 2 h at 37 °C and suitably diluted aliquots were plated on nutrient agar plates which were incubated at 37 °C for 18 h. Colonies formed were counted and percentage bacteria killed was determined.

2.4. *Circular dichroism (CD)*

Spectra were recorded in HEPES buffer (pH 7.4) with and without 2.5 mM DTT and 12 mM sodium dodecylsulfate (SDS) micelles on a JASCO J-715 spectropolarimeter at 25 °C, using a quartz cell of 1 mm path length. Data are represented as mean residue ellipticities. Peptide concentrations were 50 μM for Phd1, Phdr1, Phd3, Phdr3 and 100 μM for Phd2 and Phdr2 in buffer. The concentrations for Phd1–3 in SDS micelles were same as in buffer. Spectra were corrected for solvent contribution.

2.5. *Transmission electron microscopy (TEM)*

Bacteria were incubated with peptides at 50% LC as described above and centrifuged at 1500 × *g* for 3 min. The pellet was fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 3 h at 4 °C. After fixation, the pellet was washed thrice with 0.1 M phosphate buffer. The samples were then post-fixed with 1% (v/v) osmium tetroxide in 0.1 M phosphate buffer for 2 h. Fixed samples were washed thoroughly with phosphate buffer following which they were dehydrated through a series of acetone gradients. Dehydrated samples were passed through propylene oxide and infiltrated with epoxy resin overnight. Samples were then embedded in pure epoxy resin and dried at 60 °C for 72 h. Sections were obtained using a Reichert Ultracut E Microtome and were stained with 2% (v/v) uranyl acetate and Reynold's lead citrate. Sections were observed in a JEOL 100 CX electron microscope at 80 KV. Control samples without incubation with peptides were made in the same manner.

3. Results

The primary structures of HBD-1–3 indicate that there are considerable variations in the net charge particularly between

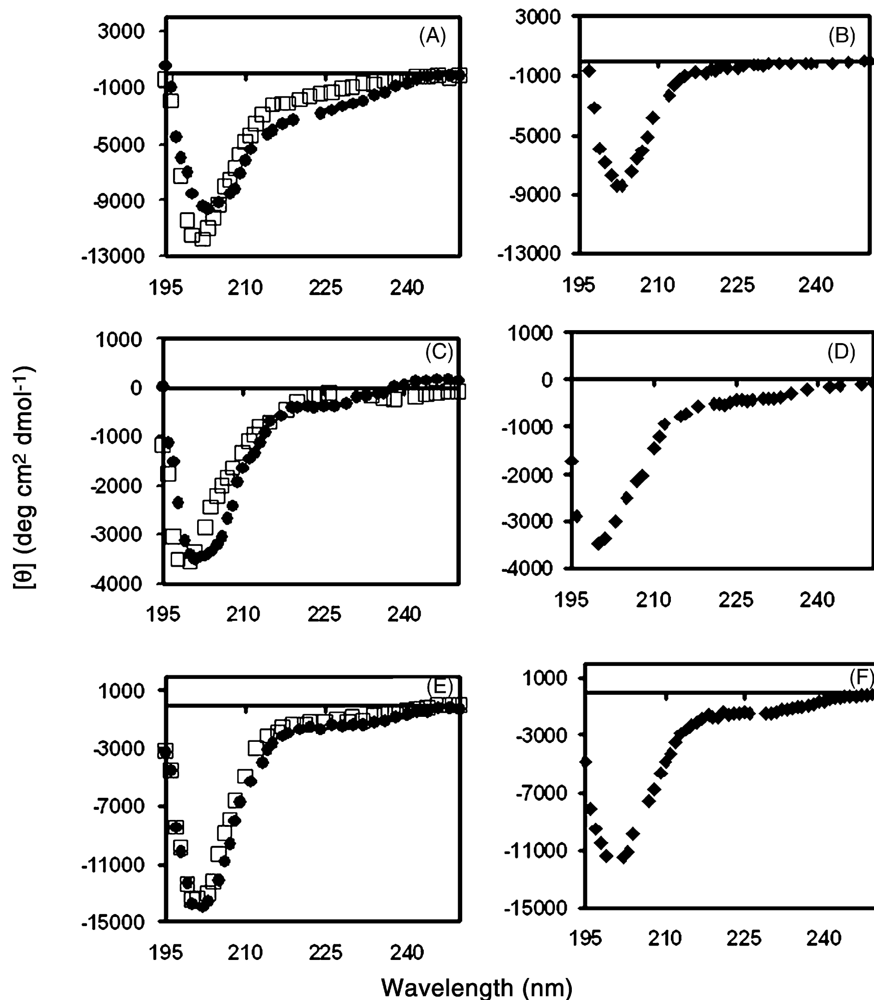

Fig. 4 – CD spectra of HBD analogs. (A), (C) and (E), Phd1, Phd2 and Phd3, respectively, in 5 mM HEPES buffer pH 7.4 and 12 mM SDS; (B), (D) and (F), Phdr1, Phdr2 and Phdr3, respectively, in 5 mM HEPES buffer pH 7.4 with DTT. Key: (□) buffer, (●) SDS and (■) with DTT.

HBD-1, 2 and 3 (Fig. 1). They have net positive charges of +4, +6 and +11, respectively. Cationic residues occur predominantly after the third cysteine in HBD-1 and 2. Nine out of the 13 cationic residues are present after Cys^3 in HBD-3. Two β-strands occur after the third cysteine. There are only minor differences in the tertiary structures of the three HBDs [3,13,14,26,27]. The peptides chosen for the study, particularly Phd1 and Phd2 span the second and third β-strands in HBD-1 and 2 with a net positive charge one more and one less than the parent peptides, respectively. Phd3 also spans the region, which has the two β-strands but has a net positive charge of +7 as compared to +11 in HBD-3. The single disulfide bond in the peptides corresponds to the disulfide between Cys^3 and Cys^6 in the parent peptides.

The antibacterial activities of Phd1–3 are shown in Table 1. All the peptides exhibited antibacterial activities against Gram-negative and Gram-positive bacteria. Phd1 is slightly more active as compared to Phd2 and Phd3 against *E. coli*, whereas Phd3 is more active as compared to Phd1 and Phd2 against *S. aureus*. The reduced forms of the peptides Phdr1–3 exhibited activity comparable to the oxidized forms against *E. coli* but were inactive up to a range of four-fold excess of LC against *S. aureus* (data not shown).

The effect of increasing concentrations of NaCl on the antibacterial activities of Phd1–3 are shown in Fig. 2. The effects on the three peptides are different. The activity of Phd1 against *E. coli* and *S. aureus* decreases with increasing concentration of NaCl and is completely abolished at 150 mM NaCl (Fig. 2A). In the case of Phd2, loss of activity against *S. aureus* is less as compared to *E. coli* up to 70 mM. At 100 mM, activity against both *E. coli* and *S. aureus* decreases considerably (Fig. 2B). The activity of Phd3 against *E. coli* is almost completely lost in the presence of 50 mM NaCl whereas against *S. aureus*, there is considerable activity at 70 mM. Complete loss of activity is observed at 100 mM NaCl (Fig. 2C and D).

The antibacterial activity of Phd1–3 in the presence of divalent cations, Mg^{2+} and Ca^{2+} was examined. Divalent cations stabilize the outer membrane of Gram-negative bacteria [36] and inhibit the antibacterial activity of defensins [21,35]. The data shown in Fig. 3 indicate that the effects of divalent cations on the antibacterial activity are variable. The

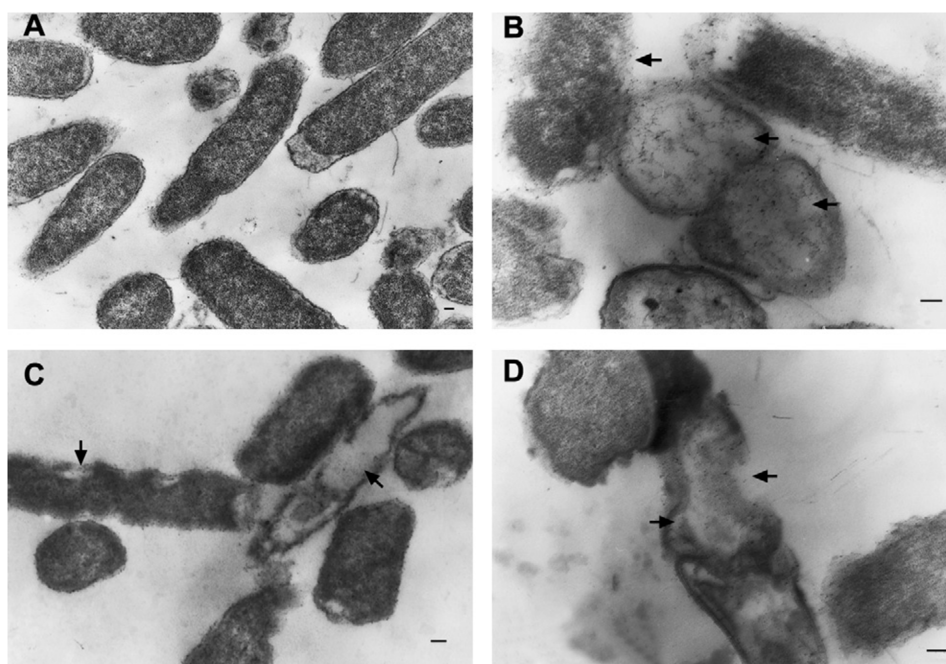

Fig. 5 – Morphological changes in *E. coli* caused by HBD analogs visualized by transmission electron microscopy. (A) *E. coli* control. Bacterial cells incubated with (B) Phd1 (C) Phd2 (D) Phd3. Bacteria incubated with peptides at 50% lethal concentration. Arrows indicate membrane rupture and contents leaked out of the cell. The bar represents 100 nm.

activity of Phd1 is inhibited marginally at 0.25 mM and ~40% killing is observed at 2 mM Mg^{2+}. The inhibition of activity is more pronounced in the presence of 2 mM Ca^{2+}. Both the cations completely inhibit the activity of Phd2 at 0.25 mM. The activity of Phd3 is partially inhibited at 0.25 mM but is completely inhibited at 2 mM of Ca^{2+} and Mg^{2+}.

Circular dichroism spectra of peptides Phd1, Phd2 and Phd3 and their reduced forms are shown in Fig. 4. In aqueous medium and SDS micelles, the spectra are similar for Phd1–3 (Fig. 4A, C, E). The spectra are suggestive of β-hairpin conformation being populated though to different extents as the negative intensity at the extrema are different. The reduced forms of the peptides (Phdr1–3) are less ordered as compared to the disulfide bridged peptides (Fig. 4B, D, F).

The effect of Phd1–3 on *E. coli* membranes was visualized by electron microscopy. The data shown in Fig. 5 indicate morphological changes in cells and extensive damage to membranes.

4. Discussion

We have investigated the antibacterial activities of synthetic peptides corresponding to the carboxy-terminal segments of HBD-1–3, which have most of the cationic residues. We observe that the synthetic peptides corresponding to the carboxy-terminal fragments of HBD-1–3, i.e. Phd1, Phd2 and Phd3, constrained by a single disulfide bridge, possess antibacterial activity against Gram-negative as well as Gram-positive bacteria. The reduced forms Phdr1–3 are active only against *E. coli*. The CD spectra of Phd1–3 in buffer and micelles are similar to the CD spectra of HBD-2 and 3 in aqueous medium [1,18]. As argued by Kluver et al. [18], it appears that peptides Phd1–3, constrained by a single disulfide bridge, have structural features similar to the native peptide. Since the reduced forms of Phd1–3 show different antibacterial spectra as compared to the disulfide constrained peptides and also greater conformational flexibility, the single disulfide bridge in the carboxy-terminal segment in human HBD-1–3 appears to play an important role in determining structure and antibacterial activity. The parent peptides HBD-1 and 2 are active only against Gram-negative bacteria [9–11,28,29,33,37,42] with HBD-2 displaying considerably greater activity as compared to HBD-1. These properties differ from the antibacterial activity of the analogs although the net positive charge of the analogs Phd1 and 2 are similar to the parent peptides. Our results suggest that in both HBD-1 and 2, the amino-terminal segments contribute to the specificity towards Gram-negative bacteria. Salt inactivation of antibacterial activity is dependent on the concentration of NaCl. While Phd1 and 2 exhibit salt sensitive activities similar to the parent defensins, the loss of antibacterial activity observed in the presence of high NaCl is not observed in HBD-3 [11,15,18,39]. It is conceivable that the higher net positive charge in HBD-3 as compared to Phd3 has a role in modulating antibacterial activity in the presence of NaCl. It is possible that the distribution of net positive charge in the carboxy-terminal segment (after the Cys^3) and the amino-terminal segment up to Cys^3 is a determinant of salt sensitivity of antibacterial activity in HBDs and possibly in other defensins. HBD-4 whose antibacterial activity is attenuated by high salt [7] has a net charge of +4 in the segment after Cys^3. Although linear analogs of HBD-3, shorter in length and with less net positive charge as compared to HBD-3, exhibit high activity against *E. coli* and *P. aeruginosa*, they are inactive in the presence of high salt [15]. The

sensitivity of antibacterial activity of Phd1–3 to divalent cations indicate that the initial site of interaction with bacteria is the bacterial cell surface as observed with full-length defensins [21,35]. Electron micrographs clearly indicate that Phd1–3 damage bacterial membranes, which results in cell death.

Rabbit α-defensin NP-1 forms voltage-dependent ion channels in planar lipid bilayer membranes [16]. The structure of human α-defensin HNP-3 in the solid state has been determined in the solid state by X-ray crystallography [12]. A model for the permeabilization of the bacterial cytoplasmic membrane by forming channel composed of more than one defensin molecule has been proposed. Based on detailed X-ray analysis, it has been concluded that neither HBD-1 or 2 can be modeled to form channel forming oligomers [13,14]. Also, nuclear magnetic resonance studies suggest that there are regions in HBD-1–3 [3,26,27] and BNBD-12 [41], which are flexible and they do not form oligomers. It has been demonstrated that the oxidized form of β-defensin (Defr-1), a dimer with intramolecular disulfide bridges shows potent, broad-spectrum antimicrobial activity, which is not attenuated at high salt concentrations [5]. Detailed analysis has revealed that Defr-1 is a complex mixture of dimer isoforms with varying intra and intermolecular disulfide connectivities. In a recent study, the interaction of HBD-3 with model membranes composed of phospholipids and lipopolysaccharides have been investigated by employing different biophysical techniques [4]. The authors have proposed a model in which electrostatic forces play an important role in the association of HBD-3 with the membrane surface. The antibacterial activity shown by several variants of HBD-3 with non-native disulfide bridges and without disulfide bridges [15,18,39] as well as the carboxy-terminal segments of HBD-1–3 reported in this study, suggests that membrane perturbation is likely to occur by a "carpet-like" mechanism as proposed by Bohling et al. [4].

Strategies for the chemical synthesis of human β-defensins to yield only the native disulfide connectivities have been reported [18,19,30]. Human β-defensins are composed of greater than 30 residues. Generation of shorter active peptides without multiple disulfide linkages would be economical for possible applications as therapeutic agents. The carboxy-terminal segments of β-defensins, particularly HBD-1–3, have a majority of cationic residues present in the native defensins and the β-strands which are a conserved structural feature in β-defensins. Hence, these peptides spanning this region would be of help in delineating the various facets of the antimicrobial activities of β-defensins such as salt sensitivity and mechanisms of bacterial membrane damage and in development of therapeutic agents.

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