CHAPTER VI

STRUCTURE-FUNCTION STUDIES ON

C-TERMINAL FRAGMENTS OF

MAMMALIAN DEFENSINS
6.1 Introduction

Defensins are unique family of cysteine-rich cationic antimicrobial peptides, widely distributed in animals and plants. Invertebrate (Andreu and Rivas, 1998; Dimarcq et al., 1998; Bulet et al., 1999) and plant- (Garcia-Olmedo et al., 1998) defensins are characterized by three and four disulphide bonds respectively, and show a common structure comprising an α-helix linked to a β-sheet by two disulphide bridges (CSαβ-motif). θ-defensins are another structurally very distinct sub-family of defensins identified in the leukocytes of Rhesus macaque monkey. These variants have a cyclic peptide backbone in addition to the three-disulphide bonds (Tang et al., 1999). Mammalian defensins are quite different from the arthropod- and plant-defensins in their sequence and structure. They have been divided into α-defensins and the β-defensins (White et al., 1995), which are characterized by an anti-parallel β-sheet structure, stabilized by three disulphide bonds (Zasloff, 2002). The classification of defensins is shown in Fig. 6.1. The mammalian α-defensins and the β-defensins differ in the number of positive charges, location and connectivities of their cysteine residues, as well as in their unique consensus sequences. Some of the examples of α-defensins and the β-defensins are given in the Fig. 6.2. The α-defensins are abundant in neutrophils, certain macrophages and paneth cells of the small intestine (Ouellette and Selsted, 1996). β-defensins have been found in the epithelial cell linings of various organs and neutrophils and respiratory track of cattle (Bevins, 1994; Diamond et al., 1996). Mammalian defensins are active against bacteria and fungi especially when tested under conditions of low ionic strength, low concentrations of divalent cations and plasma proteins. Increasing concentration of salts and plasma proteins competitively inhibit the antimicrobial activity of defensins in such a manner that they depend both on the specific defensin and its microbial target. At higher concentrations, some defensins are cytotoxic to mammalian cells (Lichtenstein et al., 1986; Lichtenstein et al., 1988; Lichtenstein, 1991).

Defensins are produced constitutively and/or expressed during infection either in response to microbial products or due to pro-inflammatory cytokines (Ganz and Lehrer 1998; Lehrer and Ganz, 1999; Schroder, 1999). In addition to their antimicrobial activity,
Fig. 6.1: Classification of defensins

<table>
<thead>
<tr>
<th>Classification</th>
<th>Origin</th>
<th>Number</th>
<th>Intramolecular disulphide bridge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant defensins</td>
<td>Plant cells</td>
<td>4</td>
<td>C1–C8, C2–C5, C3–C6, C4–C7</td>
</tr>
<tr>
<td>Insect defensins</td>
<td>Insect cells</td>
<td>3</td>
<td>C1–C4, C2–C5, C3–C6</td>
</tr>
<tr>
<td>Vertebrate α Defensins</td>
<td>Mammalian leukocytes and Paneth cells</td>
<td>3</td>
<td>C1–C6, C2–C4, C3–C5</td>
</tr>
<tr>
<td>β</td>
<td>Avian and mammalian epithelial cells and keratinocytes</td>
<td>3</td>
<td>C1–C5, C2–C4, C3–C6</td>
</tr>
<tr>
<td>θ</td>
<td>Primate leukocytes</td>
<td>3</td>
<td>C1–C6, C2–C5, C3–C4</td>
</tr>
</tbody>
</table>
### Fig. 6.2: Primary sequences of mammalian α- and β-defensins

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Primary sequence</th>
<th>Antimicrobial activity spectrum</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G +ve</td>
<td>G -ve</td>
</tr>
<tr>
<td><strong>β-Defensins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BNBD-2</td>
<td>VRNHVTCRINRGFCVPLRCPGRTIQGIOTCFGPRIKCCRSW</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HBD-1</td>
<td>GLGHRSDHNVCVSSGGCQLSACPIFTKIQTGYRCAKCCK</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HBD-2</td>
<td>GIGDPVTCLKGAIHCPVRKQITGCGLPGTCKCCRK</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HBD-3</td>
<td>GIIINTQKYYCRVRRGCARLSCLRKEEQIGKSTRGRKCCRRK</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HBD-4</td>
<td>RICGTYGTCRCKKRSCQREYRIGRPNCYACCLRKWDESLRNRTKP</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Structural features: 3 disulphide bonds, C1-C5, C2-C4, C3-C6, β-sheet and α-helix

<table>
<thead>
<tr>
<th><strong>α-Defensins</strong></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NP-2</td>
<td>VVCAACRRLACPLERRAGFCRRGRIHPLCCRR</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>HNP-1</td>
<td>ACYCRPACIAGERRYTQYQGLRALWAFCC</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HNP-2</td>
<td>ACYCRPACIAGERRYTQYQGLRALWAFCC</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>HD-5</td>
<td>ATCYCRTGRCATRESLSGVCEISGRLYRLCCR</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

Structural features: 3 disulphide bonds, C1-C6, C2-C4, C3-C5, β-sheet

Raj and Dentino, 2002
they play a role in adaptive immunity. Human neutrophil derived α-defensins (HNPs) are chemotactic for human monocytes (Territo et al., 1989) and T cells (Chertov et al., 1996). Human, rabbit and guinea pig α-defensins, as well as human β-defensins (HBD2), induce the activation and degranulation of mast cells, that results in the release of histamine and prostaglandin D2 (PGD2) (Befus et al., 1999; Niyonsaba et al., 2001).

Solution structures of a few α- and β-defensins have been determined by 2D NMR (Zhang et al., 1992; Pardi et al., 1988; Zimmermann et al., 1995; Bonmatin et al., 1992) and also high-resolution crystal structures by X-ray crystallography (Hoover et al., 2001; Hoover et al., 2000). These data reveal that N- and C-terminal residues form the polar top, and the exposed surfaces of the β-hairpins form a hydrophobic bottom of the amphiphilic structure. The polar and apolar faces of the β-sheet structure show distinct differences in the amphiphilicity of α-, β-, plant and insect defensins (White et al., 1995). The common structural feature is a hydrogen-bonded pair of anti-parallel β-strands connected by a short turn to form a β-hairpin, comprising the 15 or so residues at the C-terminal end (White et al., 1995).

Studies using the synthetic analogs of defensins could play a significant role in understanding the mechanism of action of defensins and their microbial specificity. Synthetic approach would permit the design of smaller fragments, which would aid in understanding structure-function correlation. A common conformational feature that is apparent in all the defensin structures is a β-hairpin comprising of ~18-20 amino acids spanning approximately two thirds of the C-terminal segment (Zimmermann et al., 1995; Hoover, et al., 2000; Hoover et al., 2001; Sawai et al., 2001). Earlier studies in CCMB indicated that the C-terminal fragments of BNBD-12 and NP-2 showed antibacterial activity (Mandai et al., 2002a; Mandal et al., 2002b; Thennarasu and Nagaraj, 1999). The studies on β-defensin, BNBD-12, (Mandal et al., 2002a) also revealed that the three disulphides are not essential for manifestation of antibacterial activity, as two and single disulphide variants also exhibited activity. Even the arrangement of all the three-disulphide bonds when present as in the native peptides does not seem to be necessary for antibacterial activity. This has led to design and synthesis of C-terminal fragments of bovine and human β-defensins for structure-function studies and to understand the
mechanism of action. This chapter is sub-divided into two sections as VI A and VI B. The synthesis and structure-function studies on C-terminal fragments of BNBD-2 and C-terminal fragments of human β-defensins are described in Chapter VIA and VIB respectively.
VI (A):

C-Terminal Fragments of Bovine β-Defensin-2 and its Analogs

BNBD-2 is a member of a family of 13 structurally related β-defensin peptides isolated from bovine neutrophils. Detailed analysis of high resolution protein crystal structures with respect to β-hairpin conformation have revealed that amino acids like G and P have high propensity to occur in the loop region of β-hairpin structures (Sibanda et al., 1989; Gunasekaran et al., 1997; Blanco et al., 1998). Recent studies have established that the D-proline (DP) and glycine i.e. (DPG) sequence promotes β-hairpin nucleation in peptides (Haque et al., 1994, 1996; Haque and Gellman 1997; Karle et al., 1996; Stanger and Gellman, 1998; Das et al., 1998). We have investigated the effects of introducing DPG in the C-terminal 18-38 segment of BNBD-2 on the conformation, antibacterial and hemolytic activities and interaction with model membranes. The primary sequences of the designed synthetic peptides are shown in Fig. 6A.1.

6A.2 Methods

6A.2.1 Peptide Synthesis: Cyclic and linear peptides were manually synthesized by solid-phase methods using HMPA resin by Fmoc chemistry and were cleaved from the resin as described in Materials and Methods, Chapter II. Peptides were purified by HPLC on a reversed phase C-18 column, using gradients of solvents: A: 0.1% TFA in H2O, B: 0.1% TFA in ACN. Purified peptides were characterized by MALDI mass spectrometry on a Kratos PC-Kompact MALDI 4VI.102 mass spectrometer using recrystallized α-cyano-4-hydroxycinnamic acid as matrix.

6A.2.2 Antibacterial activity: Bacterial strains used were E. coli 160.37, S. aureus (ATCC 8530) and P. aeruginosa (NCTC 6751). Lethal concentration (LC) of the peptide was determined as described in Materials and Methods, Chapter II. To determine the effect of salt on antibacterial activity, 150 mM NaCl was included in the incubation buffer. E. coli 160.37, S. aureus (ATCC 8530) and P. aeruginosa (NCTC 6751) were
Fig 6A.1: Amino acid sequence of BNBD-2 and synthetic peptides. Peptides PB1L-P4L (in parentheses) are linear forms of PB1-PB4 where the cysteine side chain protecting group Acm has been retained.
incubated with representative peptides PB1 and PB1L at their 50% LC and sections for transmission electron microscopy studies were prepared as described in Materials and Methods, Chapter II.

6A.2.3 Hemolytic activity: The hemolytic activity was determined against rat erythrocytes as described in Materials and Methods, Chapter II.

6A.2.4 Estimation of released histamine from mast cells: Mast cell isolation (Befus et al., 1999) was done as follows: Normal rats were anaesthetized, and 15 ml of cold Tyrode solution containing 12 mM HEPES and 1% BSA, pH 7.3 (HTB), were injected into the peritoneal cavity. The abdomen was massaged, and the fluid was collected and centrifuged for 5 min at 180 X g at 4°C. Peritoneal exudates were layered on a 30%/80% gradient of percoll and centrifuged for 20 min at 220 X g at 4°C. The cell pellet was washed twice and examined for mast cell purity (>98 %) and viability (97%) by trypan blue exclusion.

Highly enriched peritoneal mast cells (30,000) and peptides to be used were preincubated separately at 37°C for 5 min. Peptides were then added to the mast cell-suspension and incubated at 37°C for 10 min. The reaction was terminated by the addition of 1 mL of cold HTB and was centrifuged at 4°C to separate the supernatant and cell pellet. After the pellets were brought to 1.5 mL using HTB, the samples were boiled for 10 min to release the cell-associated histamine and destroy histaminase activity. After precipitation of proteins using TCA, histamine levels were measured in both supernatant and pellet fractions by fluorometric assay using OPA on F4500 Spectrofluorimeter. Histamine release was expressed as a percentage of the cellular histamine content as calculated by the formula: (histamine in supernatant)/(histamine in supertanant and pellet) X 100.

6A.2.5 Circular Dichroism studies: Spectra were recorded in HEPES buffer (pH 7.4), TFE and SDS micelles on a JASCO J-715 automatic recording spectropolarimeter as described in Materials and Methods, Chapter II. Peptide concentrations were 100 µM in buffer and SDS micelles and 50 µM in TFE.
6A.2.6 Binding of peptides to micelles and model membranes: Binding was assessed by monitoring the fluorescence of tryptophan in the presence of micelles and lipid vesicles. The excitation monochromator was set at 280 nm. The slit width was 5 nm. All the measurements were carried out on a F 4500 Hitachi Fluorescence Spectrometer. Large unilamellar vesicles were prepared by extrusion through polycarbonate filters (100 nm pore filter) in a mini extruder as described by MacDonald et al., (1991). Resonance energy transfer experiments were carried out with lipid vesicles doped with 5 mol% of dansyl PE.

6A.2.7 Permeabilization studies with model membranes: The ability of peptides to permeabilize model membranes was examined by monitoring the dissipation of diffusion potential set up in lipid vesicles by valinomycin. Lipid vesicles with entrapped K⁺ were diluted into K⁺ free buffer followed by addition of cyanine dye diS-C₃-(5) (Sims et al., 1974), valinomycin and peptides. The excitation and emission wavelengths were 620 and 670 nm respectively. The percentage of fluorescence recovery F was calculated by using the equation

\[ F = \left( \frac{F_t - F_o}{F_r - F_o} \right) \times 100 \]

where, \( F_1 \) is the fluorescence observed after addition of peptide at time \( t \); \( F_o \) is the fluorescence after addition of valinomycin and \( F_r \) is the total fluorescence prior to the addition of valinomycin.

6A.3 Results

6A.3.1 Choice of peptides: Peptide PB1 corresponds to the sequence in the C-terminal fragment of BNBD-2 spanning the region linked by a disulphide bond between cysteines C₃ and C₆. Cysteine C₅ which is connected to C₁ was not included, and C₃ which is connected to C₂ was replaced by I. This segment also has six of the nine cationic amino acids present in BNBD-2. In PB2, residue I was replaced by D-proline (DP) with a view to examine the propensity of the peptide to adopt a well ordered β-hairpin structure. In peptide PB3, the GP sequence at positions 14, 15 was reversed to PG and the P in them were replaced by DP in PB4. Oxidation of all the peptides was carried out in 20%
aqueous DMSO as described, to obtain cyclic peptides. Linear form of the peptides PB1L-PB4L containing cysteines protected by Acm were also generated. All the eight peptides (linear and cyclic) were used for studies.

6A.3.2 Antibacterial and hemolytic activities: The antibacterial activities of the peptides (cyclic as well as their linear counterparts) are summarized in Table 6A.1. With the exception of PB3 all the other peptides are active at comparable concentrations against *E. coli*. PB3 is ~2.5 fold less active. In fact, PB3L is more active than PB3. With the exception of PB2, the other peptides exhibit slightly lower activity against *P. aeruginosa*. The activities against *S. aureus* are comparable. All the peptides were inactive against bacteria when 150 mM NaCl was present in the buffer. About 5-6 fold more of peptide was necessary to inhibit growth in the presence of salt. However, in the presence of salt, the peptides were not effective in killing *P. aeruginosa* even at high concentrations. The hemolysis was determined against rat erythrocytes, and a varying extent of lysis was observed. Maximum lysis was observed with PB1 and PB1L. However, the concentrations at which ~53% lysis was observed was 10 fold higher than the lethal concentration (LC). Except PB2L which exhibited 54% lysis at 40 μM, the other peptides do not exhibit marked lytic activity even at ~10 fold concentration higher than the LC.

The effect of PB1 and PB1L on *E. coli*, *P. aeruginosa* and *S. aureus* was examined by transmission electron microscopy (Fig.6A.2). At 50% lethal concentration, distinctive changes in morphology were clearly observed. TEM revealed wrinkled bacterial surfaces and clustered cytoplasmic contents in the cells. Membrane integrity is compromised as indicated by arrows. In *S. aureus*, vacuoles in the cytoplasm were discernible and mesosome-like structures were seen in peptide-treated cells.

6A.3.3 Histamine secretion from mast cells: The abilities of BNBD-2 analogs to induce histamine secretion from purified rat PMC were studied. The analogs induced a maximum of 20-50% histamine release as summarized in Table 6A.1. The membrane
Table 6A.1: The antibacterial and hemolytic activities and % histamine release from mast cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Antibacterial Activity (µM)</th>
<th>% RBC Lysis</th>
<th>%Histamine Release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> (<em>+/− NaCl</em>)</td>
<td><em>S. aureus</em> (<em>+/− NaCl</em>)</td>
<td><em>P. aeruginosa</em> (<em>+/− NaCl</em>)</td>
</tr>
<tr>
<td>PB1</td>
<td>4/16</td>
<td>4/16</td>
<td>6/ NA</td>
</tr>
<tr>
<td>PB1L</td>
<td>4</td>
<td>5</td>
<td>5/ NA</td>
</tr>
<tr>
<td>PB2</td>
<td>4/16</td>
<td>4/16</td>
<td>4/ NA</td>
</tr>
<tr>
<td>PB2L</td>
<td>4</td>
<td>5</td>
<td>4/ NA</td>
</tr>
<tr>
<td>PB3</td>
<td>10/16</td>
<td>4/16</td>
<td>7/ NA</td>
</tr>
<tr>
<td>PB3L</td>
<td>10</td>
<td>6</td>
<td>5/ NA</td>
</tr>
<tr>
<td>PB4</td>
<td>4/16</td>
<td>4/16</td>
<td>5/ NA</td>
</tr>
<tr>
<td>PB4L</td>
<td>4</td>
<td>6</td>
<td>10/ NA</td>
</tr>
</tbody>
</table>

NA- Not active

* 150 mM NaCl
Fig. 6A.2: Effect of peptides on bacteria visualized by transmission electron microscopy: (a) *E. coli* control (b, c) after incubation with PB1L and PB1 respectively. (d) *P. aeruginosa* control (e, f) after incubation with PB1L and PB1 respectively. (g) *S. aureus* control (h, i) after incubation with PB1L and PB1 respectively. Arrows indicate breaks in the membrane. The bar represents 100 nm. Bacteria were incubated with peptides at 50% lethal concentration as described in Materials and Methods.
rupture and degranulation of mast cells with the peptides was also observed by electron microscopy (Fig. 6A.3).

**6A.3.4 Conformation of peptides:** The conformations of linear and cyclic peptides were examined in aqueous medium and in TFE and SDS micelles, which provide a membrane-like environment (Hoyt et al., 1991; Watson et al., 2001; Venkatraman et al., 2001; Krishna et al., 2002). The CD spectra of linear and disulphide bridged peptides are shown in (Fig.6A.4). In buffer, the linear peptides show spectra characteristic of a large fraction of peptide populating unordered conformation. In TFE, PB1L shows negative minima at ~205 and 225 nm with a crossover at ~200 nm. The spectrum is similar to defensins with three disulphide bridges (Rao et al., 1992; Dawson et al., 2000; Circo et al., 2002; Weis et al., 2002) populating helical and β-hairpin conformation (Blanco et al., 1994; Blanco et al., 1995; Viguera et al., 1996). Introduction of DPG and switching of GP to PG appears to favour β-hairpin conformation even in the linear peptides. The spectra of peptides in SDS micelles also suggest β-hairpin conformation.

Spectra of cyclic peptides PB1, PB2 and PB4 indicate β-hairpin formation even in aqueous medium. Peptide PB3, where GP has been switched to PG, is unordered. In TFE and SDS micelles, spectra of PB2, PB3 and PB4 indicate β-hairpin structures whereas there appears to be a mixed population of helical and β-hairpin structures in the case of PB1. CD spectra indicate that introduction of DPG favours β-hairpin conformation even in aqueous medium. When GP to PG change is introduced, β-hairpin structure is observed in TFE and SDS micelles.

**6A.3.5 Interactions with micelles and model membranes:** The binding of peptides to SDS micelles and lipid vesicles composed of POPC and POPE/POPG (3:1), were examined by monitoring tryptophan fluorescence. All peptides bind to SDS micelles in a similar manner (Fig.6A.5).

In buffer, all the peptides showed a $\lambda_{\text{max}}$ of 355 nm indicating exposure of the fluorophore to aqueous environment. When the peptides were titrated with increasing
Fig. 6A.3: Effect of BNBD-2 analogs on mast cell degranulation, visualized by transmission electron microscopy. (A) Control mast cell, (B) Incubation with PBlL (C) Incubation with PB1. (Magnification 2000).
Fig. 6A.4: Circular dichroism spectra of linear and cyclic peptides. Spectra were recorded in 5 mM HEPES buffer, pH 7.4, TFE and in 10 mM SDS micelles. Key (□) buffer, (●) SDS micelles; (△) TFE. Concentrations of peptides in buffer and SDS micelles were 100 μM and 50 μM in TFE.
Fig. 6A.5: Interaction of linear and cyclic peptides with SDS micelles. Different concentrations of SDS were added to a fixed concentration of peptide (4 μM). Tryptophan fluorescence was monitored. F and F₀ correspond to fluorescence intensities at λ_max in the presence and absence of SDS. Key (○) linear peptides; (●) cyclic peptides.
concentration of POPC vesicles, no shift in the position of $\lambda_{\text{max}}$ was observed. However, there was quenching of fluorescence with increasing lipid concentration for both linear and cyclic peptides (Fig.6A.6). The quantum yield in the absence of lipids varies considerably in peptides PB3 and PB3L. The quenching was also observed at high lipid/peptide ratios of 1000:1.

The fluorescence spectra of the peptides were examined in the presence of POPE/POPG (3:1) vesicles (Fig.6A.7). The spectra were recorded at lipid/peptide ratios where maximal changes were observed. The spectra indicate variation in the fluorescence behavior of the peptides. A small blue shift is observed for all the linear peptides. In PB1L-PB3L, there is enhancement in intensity, while there is a slight quenching in PB4L. Peptides PB1 and PB2 show blue shifts with very little change in intensity whereas PB3 and PB4 show quenching of fluorescence. The location of tryptophan in the peptides, in POPE/POPG vesicles, appears to be different from POPC vesicles. The interaction of PB1 and PB1L with POPE/POPG vesicles, was further examined by monitoring energy transfer between tryptophan and dansyl fluorophore in lipid vesicles doped with dansyl-PE (Fig.6A.8). Significant energy transfer was observed indicating that the linear and disulphide-constrained peptides bind to POPE:POPG vesicles. Since the antibacterial activity of all the peptides is compromised by high salt, energy transfer in the presence of salt was examined. Considerable reduction in energy transfer was observed (data not shown).

We examined the ability of the peptides to permeabilize model membranes by monitoring the dissipation of diffusion potential created by valinomycin (Sims et al., 1974) (Fig.6A.9). It is evident that all the peptides permeabilize POPE/POPG lipid vesicles in the absence of NaCl, while they do not permeabilize POPC vesicles.

6A.4 Discussion

The $\beta$-hairpin structure at the C-terminus is conserved in $\alpha$- and $\beta$-defensins. The $\beta$-hairpin structure is stabilized by the three disulphide bridges. In $\alpha$- and $\beta$-defensins all
**Fig. 6A.6:** Interaction of peptides with POPC (LUVs). Different concentrations of POPC (LUVs) were added to a fixed concentration of peptide (4 μM). The decrease in fluorescence at $\lambda_{\text{max}}$ is plotted against lipid concentration. Key: (●) cyclic peptides; (○) linear peptides.
Fig. 6A.7: Interaction of peptide with POPE/POPG (3:1) LUVs. Fluorescence spectra of peptide in buffer (thin line) and in the presence of POPE/POPG lipid vesicles (thick line). A fixed concentration of peptide (4 μM) was titrated with increasing concentrations of lipid vesicles. The data presented are maximal changes observed at a lipid/peptide ratios of 1:15.
Fig. 6A.8: Interaction of peptide with POPE/POPG (3:1) LUVs doped with dansyl-PE. Increasing concentrations of peptide were added to the lipid vesicles (50 μM). Spectra were obtained with the excitation wavelength at 280 nm. Key: (dark line) lipid blank; (thin lines) in the presence of peptides at peptide/lipid ratios of (1) 1: 60, (2) 1: 30, and (3) 1: 15.
Fig. 6A.9: Maximal dissipation of diffusion potential in POPE/POPG vesicles induced by peptides. The peptides were added to LUV (25 μM) in K⁺-free buffer pre-equilibrated with the fluorescent dye diS-C₃(5)-C₅ and valinomycin. Fluorescence recovery was measured 10 min after peptides were mixed with the vesicles. Key (○) linear peptides; (●) cyclic peptides.
the three disulphide bridges as well as the disulphide connectivities observed in the native sequence, are not essential for manifestation of antibacterial activity (Thennarasu and Nagaraj, 1999; Mandal and Nagaraj, 2002a; Mandal et al., 2002b). In a recent report, it has been demonstrated that a synthetic peptide corresponding to an intragenic polymorphic form of human β-defensin 1, where the disulphide connectivities are C1-C2 and C3-C5 (this form has 5 cysteines instead of 6 normally present in HBD-1) shows antibacterial activity (Circo et al., 2002). We have attempted to stabilize the β-hairpin conformation in the peptide segment corresponding to the C-terminal segment of BNBD-2 by introducing DPG and switching the GP sequence to PG.

The peptides showed antibacterial activities as well as salt inactivation like their parent peptide. The lethal concentrations do not appear to directly correlate with the conformational propensities of the peptides, as even the linear peptides exhibited the activity. All the peptides showed low hemolytic activities except PB1. It is possible that the higher hemolytic activity of PB1 is due to the presence of peptides populating helical conformation. The salt-sensitive antibacterial activity of peptides corresponding to the C-terminal segment of BNBD-2 suggests that this region is perhaps the determinant of antibacterial activity in β-defensins. Peptides corresponding to the C-terminal region of defensin could be useful in determining the mechanism of salt inactivation of the antibacterial activity. We observed morphological alterations in cells treated with representative peptides PB1 and PB1L. E. coli and P. aeruginosa showed presence of striking, electron-dense deposits in the cytoplasm, outer membrane rupture and wrinkled bacterial shapes. Mesosome-like structures were seen in S. aureus but not in E. coli. The EM data indicates that there is a catastrophic effect on the cytoplasmic organization in bacterial cells treated with defensin analogs compared to control cells.

There is increasing evidence that defensins also participate in other aspects of innate immunity such as activation of mast cells and induction of cytokines (van Wetering et al., 1999; Lehrer and Ganz, 1999; Lehrer and Ganz, 2002) and have a role in adaptive immunity (Yang, De et al., 2002). In contrast to α-defensins, β-defensins are expressed in epithelial tissues to defend epithelium from infection (Fulton et al., 1997;
Goldman et al., 1997; Valore et al., 1998). Mast cells are recognized as an important component in inflammation and immune surveillance mechanisms, and their products are shown to be important in inflammatory reactions. The mast cell granule products increase neutrophil influx (Echtenacher et al., 1996; Malaviya et al., 1996). Defensin-induced mast cell degranulation and IL-8 production at inflammatory sites would promote the recruitment and accumulation of neutrophils (Oppenheim et al., 1991; Baggiolini et al., 1994; Malaviya et al., 1996; Echtenacher et al., 1996). Peptide antibiotics such as HBD-1, HBD-2 and LL-37 expressed in epithelial tissues were shown to release histamine and/or generate PGD\(_2\) in a G protein-dependent manner (Niyonsaba et al., 2001) from rat peritoneal mast cells that belong to the connective tissue-type mast cells (Gibson and Miller, 1986). Since the C-terminal fragments i.e. \(\beta\)-hairpin forming segment of defensins exhibited antibacterial activity, we have examined the interaction of BNBD-2 analogs with mast cells and found that they release the 20-50% histamine.

We have observed the incorporation of \(^{31}\)PG and switching of GP to PG appears to stabilize \(\beta\)-hairpin conformation (Haque et al., 1994, 1996; Karle et al., 1996; Haque and Gillman, 1997; Stanger and Gellman, 1998; Das et al., 1998). While this stabilization does not result in enhanced antibacterial activity, there is a reduction in the hemolytic activity. A rigid \(\beta\)-hairpin conformation and disulphide bond does not appear to be essential for antibacterial activity in synthetic peptides spanning the C-terminal segment of BNBD-2.

Rabbit \(\alpha\)-defensin NP-1 has been shown to form voltage-dependent ion permeable channels in planar lipid bilayers (Kagan et al., 1990; Fujii et al., 1993). Human and rabbit \(\alpha\)-defensins bind to negatively charged model membranes in preference to zwitterionic model membranes (Wimley et al., 1994; Lohner et al., 1997; Hirstova et al., 1996, 1997) and cause membrane permeabilization by forming multimeric pores (Wimley et al., 1994). Since the BNBD-2 segment and analogs described in Fig.6A.1 have six of the nine cationic amino acids present in the parent peptide, their interactions with model membranes were investigated.
With SDS micelles, enhancement in fluorescence was observed. There was quenching of tryptophan fluorescence seen in the presence of POPC lipids. It is unlikely that quenching is due to intermolecular interactions as quenching was also observed at high lipid/peptide ratios of 1000:1. It has been suggested that intermolecular quenching of tryptophan fluorescence can occur due to quenching by peptide bonds (Sillen et al., 2000; Adams et al., 2002). Single phosphate groups from phospholipids have been reported to have the ability to quench fluorescence of tryptophan in peptides (Kroon et al., 1990; Yeager and Feigenson, 1990; van Heusden et al., 2002). We attribute the quenching of tryptophan fluorescence in all the peptides as a result of localization of the tryptophan near the phosphate of the head group. In a recent study, the quenching of tryptophan fluorescence in the antibiotic nisin has been attributed to phosphate groups (van Heusden et al., 2002).

Binding of peptides with POPE/POPG vesicles reached a maximum at a peptide/lipid 1:15 and increase in fluorescence or shift in emission maximum was marginal as compared to any helical peptides. Significant energy transfer between tryptophan and dansyl fluorophore was observed by monitoring the interaction of PB1 and PB1L with POPE/POPG vesicles doped with dansyl PE. This shows that linear and disulphide-constrained peptides bind to POPE/POPG vesicles. The energy transfer was reduced when NaCl was included in the buffer suggesting that the peptides do not bind to lipid vesicles in the presence of high salt. Hence, loss of antibacterial activity in the presence of salt would arise due to the inability of the peptides to associate with bacterial membranes that are rich in POPE and POPG.

The peptides PB1-PB4 as well as their linear analogs cause membrane destabilization of POPE: POPG (3:1) vesicles in the absence of salt, which is evident from the dissipation of diffusion potential created by valinomycin. Similar membrane destabilization was not observed with POPC vesicles, which could be the reason for the relatively low hemolytic activity of these peptides. The experiment was carried out in the absence of salt since antibacterial activity was inhibited in the presence of salt. The absence of salt in the external medium would lead to membrane tension induced by
osmotic swelling of vesicles. Osmotically induced membrane tension in lipid vesicles modulates membrane permeabilization of lytic peptides (Polozov et al., 2001). In the present case, permeabilization of the lipid vesicles is unlikely to occur if the peptides did not have the property of destabilizing membranes. Binding of linear and cyclic peptides to POPE/POPG (3:1) lipid vesicles causes membrane destabilization resulting in membrane permeabilization.

The peptides interact with zwitterionic and negatively charged vesicles but with different orientations. However, permeabilization is observed only with POPG containing vesicles, consistent with the observations on α-defensins (Wimley et al., 1994; Lohner et al., 1997; Hirstova et al., 1996, 1997). Fluorescence data suggest that the peptides are oriented on the surface of the bilayer and cause membrane destabilization due to electrostatic interactions rather than by a mechanism involving pore-formation as proposed for magainin and other membrane-active antibacterial peptides (Matsuzaki, 1998; McElhaney and Prenner, 1999). Hence, short peptides corresponding to the C-terminal segments of defensin with β-hairpin conformation are useful to understand the mechanism of antibacterial activity and could have potential as therapeutic agents, as they would be convenient to synthesize and also provide greater resistance to proteolysis.

6A.5 Summary

- The C-terminal fragment of bovine β-defensin-2, which has the two β-strands, has been chosen for designing the analogs.

- The sequence, D-Proline-Glycine (DPG) favors nucleation of β-hairpin structure. Thus, two analogs, one with DPG at central portion and another with two DPG sequences near N-and C-terminal ends were generated, each with a single disulphide bond. The corresponding linear analogs were also generated.

- The linear and cyclic analogs of BNBD-2 showed antibacterial activity but the hemolytic activity was not very significant. Transmission electron microscopy
studies show morphological changes in bacteria when treated with peptide analogs.

- These peptide analogs became inactive against bacteria in the presence of 150 mM NaCl like parent peptide BNBD-2.

- Circular dichrosim studies indicated that introduction of DP in the sequence renders it to adopt ordered conformation in aqueous medium.

- The binding studies with POPC, POPE/POPG vesicles and SDS micelles show that these peptides bind to membranes and membrane permeabilization is the mechanism of their action.

- The fluorescence energy transfer with DNS group showed that peptide binds to POPE/POPG vesicles and binding is affected in presence of NaCl.

- The peptides release histamine from mast cells.

- The entire BNBD-2 defensin parent sequence does not appear to be essential for manifestation of antibacterial activity.
C-Terminal Fragment of Human β-Defensins

Six α-defensins (neutrophil peptides HNP-1 to HNP-6) and four β-defensins (HBD-1 to HBD-4) have been currently identified from humans (Hoover et al., 2003). They are salt-sensitive and differ substantially in their antimicrobial specificities. For e.g. HBD-2 is active against Gram-negative bacteria and the fungus Candida albicans, but has little to no activity against Gram-positive bacteria (Harder et al., 1997). HBD-4 is more active against S. carnosus and P. aeruginosa but not active on E. coli, Saccharomyces cerevisiae, and S. aureus in conventional dilution assays (Garcia et al., 2001). On the other hand HBD-1 and HBD-3 display broad antibacterial activity against Gram-negative and Gram-positive bacteria (Ganz et al., 1985; Bensch et al., 1995; Harder et al., 2001). The antibacterial activity of HBD-3 unlike HBD-1 and HBD-2 is insensitive to salt. The differences in the antibacterial activities between HBD-1, HBD-2 and HBD-3 cannot be easily ascribed to individual residues in their sequence. Since most of the charged residues in HBD-1 and 2 occur after C³ (third cysteine from N-terminal), peptides corresponding to the region after C³ with same number of residues and charges (+5) were synthesized and their antibacterial properties examined. HBD-3 unlike HBD-1 and HBD-2 has charged residues between the N-terminus and C³. For the sake of comparison, the region after C³ in HBD-3 was synthesized. The designed synthetic peptide analogs are given in Fig. 6B.1.

6B.2 Methods

6B.2.1 Peptide Synthesis: Peptides were manually synthesized by solid-phase methods using HMPA resin employing Fmoc chemistry and were cleaved from the resin as described in Materials and Methods, Chapter II. They were purified by HPLC on a reversed phase C-18 column using gradients of solvents: A: 0.1% TFA in H₂O, B: 0.1% TFA in ACN and characterized by MALDI mass spectrometry on a Kratos PC-Kompact.
**Fig 6B.1:** Primary sequence of human β-defensins analogs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Primary sequence</th>
<th>Net charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBD-1</td>
<td>GLGHRSDHYNCVSSGGQCLYSAACPIIFTKIQGTCTYRGKAKCK</td>
<td>+5</td>
</tr>
<tr>
<td>PH1</td>
<td></td>
<td>+5</td>
</tr>
<tr>
<td>HBD-2</td>
<td>GIGDPVTCLKSGAICHPVFCPRRYQIGTCLGPLGTKCKK</td>
<td>+6</td>
</tr>
<tr>
<td>PH2</td>
<td></td>
<td>+5</td>
</tr>
<tr>
<td>HBD-3</td>
<td>GIINTLQKYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK</td>
<td>+11</td>
</tr>
<tr>
<td>PH3</td>
<td></td>
<td>+7</td>
</tr>
</tbody>
</table>
MALDI 4VI.102 mass spectrometer using recrystallized α-cyano-4-hydroxycinnamic acid as matrix.

6B.2.2 Antibacterial activity: The antibacterial activity of the peptide was examined as described in Materials and Methods, Chapter II. Bacterial strains *E. coli* 160.37 and *S. aureus* (ATCC 8530) were used. The lethal concentration (LC) was determined. To determine the effect of salt on antibacterial activity, different concentrations of NaCl were 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 peptide. Bacterial cells were incubated with peptides at their 50% LC and the sections of these cells were prepared for TEM as described in Materials and Methods, Chapter II.

6B.2.3 Hemolytic activity: The hemolytic activity was determined against rat erythrocytes as described in Materials and Methods, Chapter II.

6B.2.4 Circular Dichroism studies: Spectra were recorded in HEPES buffer (pH 7.4), TFE and SDS micelles on a JASCO J-715 automatic recording spectropolarimeter as described in Materials and Methods, Chapter II. Peptide concentrations were: (1) 50 μM PH1 in buffer, SDS micelles and TFE, (2) 100 μM PH2 in buffer, SDS micelles and 275 μM in TFE, (3) 60 μM PH3 in buffer, SDS and 30 μM in TFE.

6B.2.5 Outer membrane permeability: To evaluate outer membrane permeability, the hydrophobic fluorescent probe 1-N-phenyl naphthylamine (NPN) was used. *E. coli* MG 1655 cells were grown to late logarithmic phase in nutrient broth. The harvested cells were washed twice with 10 mM phosphate buffer pH 7.4 and diluted in the same buffer to an OD$_{600}$ of 0.025. A 1 mL aliquot of the cells containing 10 μM NPN was taken for each experiment. The excitation monochromator was set at 350 nm and emission at 420 nm. After addition of the peptide from an aqueous stock solution, the changes in fluorescence were continuously monitored with constant stirring.

6B.2.6 Inner membrane permeability: Inner membrane (IM) permeability in the presence of the peptides was assessed by using O-nitrophenyl-3D-glactoside (ONPG), a
substrate for cytoplasmic β-galactosidase enzyme. O-nitrophenol (ONP), the hydrolysis product of ONPG by β-galactosidase, is yellow in colour and absorbs maximally at 420 nm. E. coli GJ 2455 (MG 1655) (lacI:: kan recA 56), cells were grown to mid log phase in nutrient broth. The cells were diluted to an OD

\[ \text{OD}_{600} \] of 0.05 with 10 mM sodium phosphate buffer pH 7.4 containing 0.5 mM ONPG. Aliquots of this were incubated with different concentrations of peptides at 37°C. OD measurements made at 420 nm at different time intervals, which reflects ONPG influx into the cells, is taken as an indicator of the permeability status of the IM. The IM permeability was also determined in the presence of 100 mM NaCl.

Cytoplasmic membrane permeability assay with cyanine dye diSC3-5: Cytoplasmic membrane permeabilization was also determined by using the membrane potential sensitive cyanine dye diSC3-5. It distributes between cells and medium depending on the cytoplasmic membrane potential gradient which is a measure of the disruption of the electrical potential gradient across the cytoplasmic membrane of intact bacteria (Wu et al., 1999b; Wu et al., 2003). The partially outer membrane defective mutant E. coli DC2 (gift from Genetics Stock Center CGSC, Yale University) (Clark, 1984, 1985) was used so that diSC3-5 could readily reach the cytoplasmic membrane. Bacteria were grown at 37°C to mid logarithmic phase \( \text{OD}_{600} = 0.5-0.6 \). Cells were collected by centrifugation, washed with buffer (5 mM HEPES, pH 7.4, 5 mM glucose) and resuspended in the same buffer to an OD

\[ \text{OD}_{600} \] of 0.05. The cell suspension was incubated with 0.4 µM diSC3-5 until its uptake was maximal. The IM permeabilization with peptides PH1, PH2 and PH3 was determined in the presence of 20 mM NaCl and 100 mM NaCl in 1 ml cell suspension. The fluorescence reading was monitored after the addition of the peptides, in F-4500 fluorescence spectrophotometer, at an excitation wavelength of 622 nm and an emission wavelength 670 nm. The maximal fluorescence increase due to the disruption of the cytoplasmic membrane was recorded.

For control experiment, valinomycin (the K⁺ specific ionophore) was used to modulate membrane potential of the cells. The varying K⁺ concentration in the presence of valinomycin outside the cells changes the potential accordingly. This was indicated by plotting the fluorescence of diSC3-5 as a function of K⁺ concentration outside the cells.
As the concentration of K\(^+\) outside increased, the fluorescence intensity increased proportionally. A linear relationship was observed over the range 10-100 mM external KCl concentration (data-not shown). At 0.1 M KCl, the potential increase reached its maximum due to equilibration of the outside and inside K\(^+\) concentration. This result demonstrated that the partitioning of diSC3-5 between the suspension medium and cells was directly proportional to the size of the membrane potential which is maximal at 100 mM KCl.

6B.2.7 Effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP) and glucose on antibacterial activity and salt inactivation: CCCP has been used extensively in studies on the proton motive force (PMF)-dependent efflux of antibacterial substances by bacteria (Shafer et al., 1998). CCCP is a proton conductor that depolarizes the cytoplasmic membrane. The loss of efflux activity due to the depolarizing effect of CCCP can be reversed by 20 mM glucose (Ohyama et al., 1992). The determination of antibacterial activity protocol was modified slightly to test the effect of CCCP. Bacterial culture of *E. coli* MG1655 was grown in nutrient broth to mid log phase and diluted to 10\(^6\) colony-forming units in 10 mM phosphate buffer pH 7.4 containing 50 \(\mu\)M CCCP and then incubated for 20 min. Subsequently, these cells were incubated for 10 min with 20 mM glucose to reverse the effect of CCCP. Lethal concentrations of test peptide were used for checking the antibacterial activity of peptides with CCCP and glucose treated cells. For determining the effect of salt, 100 mM NaCl was included in buffer. Bacterial cells were incubated for 2h at 37\(^\circ\)C and suitably diluted aliquots were used for plating.

6B.3 Results

6B.3.1 Choice of peptides: The primary structures of human \(\beta\)-defensins 1-3 (Fig. 6B.1) show that there are considerable variations in the net charge and hydrophobicities. They have net positive charges of +5, +6 and +11 respectively. There are only minor differences in the tertiary structures of the three human \(\beta\)-defensins. Cationic residues and two out of the three \(\beta\)-strands occur predominantly after cysteine C\(^3\) in HBD-1 to 3. Peptide fragments of HBD-1 and 2 spanning the region from C\(^3\) (PH1 and PH2) have the
same number of residues and net positive charge of +5 respectively. The same segment (after cysteine C³) in HBD-3 has a net charge of +7 as compared to +11 in the parent sequence. Nine out of the 13 cationic residues are in the in C-terminal region in HBD-3. Therefore, peptides PH1, PH2 and PH3 fragments corresponding to the C-terminal segment of HBD-1, HBD-2 and HBD-3 respectively, linked by a disulphide bond between cysteine C³ and C⁶ (omitting cysteines C⁴ and C⁵) were synthesized, in order to examine their antibacterial activities and conformation.

6B.3.2 Antibacterial and hemolytic activities: The antibacterial activities of the peptides and hemolytic activities against human and rat RBCs are shown in Table 6B.1. All the peptides exhibited antibacterial activities against Gram-negative and Gram-positive bacteria. PH1 is slightly more active than PH2 and PH3 against *E. coli* whereas, PH3 is more active as compared to PH1 and PH2 against *S. aureus*. Lysis of rat and human erythrocytes was examined in isotonic NaCl and sucrose. At concentrations three times the LC against *E. coli*, the peptides lyse rat RBCs to different extents. PH1 causes maximum lysis, which is 2.5 to 3 times greater than that caused by the PH2 and PH3. In sucrose medium all these peptides exhibited greater lytic activity against rat and human RBCs. However, the extent of lysis is comparable in both sucrose and NaCl media for rat and human RBCs with fragments of human β-defensins.

The minimal threshold concentrations of NaCl required for inactivation of these peptides against *E. coli* and *S. aureus* was found to be different for each of these peptides (Fig. 6B.2). The percentage killing of bacteria for both PH1 and PH2 decreases with increasing NaCl. The activity against *E. coli* appears to be more salt-sensitive as compared to *S. aureus*, as killing to a greater extent is observed for the latter even at 70 mM NaCl. The antibacterial activity of PH3 against *S. aureus* is compromised in the presence of NaCl to a much greater extent. Against *E. coli*, 50% activity is lost at 30 mM NaCl and complete growth is observed at 50 mM NaCl. There is an abrupt loss in activity against *S. aureus* between 70-100 mM NaCl. When *E. coli* cells were treated with analogs of human β-defensins, morphological changes in the bacteria were noticed.
Table 6B.1: The antibacterial and hemolytic activities of human β-defensins analogs

<table>
<thead>
<tr>
<th>S. No</th>
<th>Peptide</th>
<th>Microorganisms</th>
<th>% RBC Lysis (at 60 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. coli LC (µM)</td>
<td>S. aureus LC (µM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td>Sucrose</td>
</tr>
<tr>
<td>1</td>
<td>PH1</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>PH2</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>PH3</td>
<td>17</td>
<td>17</td>
</tr>
</tbody>
</table>
Fig. 6B.2: Effect of NaCl on antibacterial activity of human beta defensin analogs. Open bars represent *E. coli* and dark bars represent *S. aureus*.
in EM pictures (Fig. 6B.3). In peptide treated cells, membrane was damaged and their contents leaked out.

6B.3.3 Conformation of peptides: Circular dichroism spectra of the synthesized peptides PH1, PH2 and PH3 shown in Fig. 6B.4. In aqueous medium and SDS micelles, the spectra are similar for all the peptides. The spectra are suggestive of β-hairpin conformation being populated though to different extents as the negative intensity at the extrema are different. The spectrum does not change substantially in the presence of membrane-mimicking SDS micelles compared to TFE. In TFE, the spectra of PH1 and PH3 show α-helical structure whereas the spectrum of PH2 is similar to poly-proline like structure (Ronish and Krimm, 1974).

6B.3.4 Outer membrane permeabilization: The ability of the peptides to permeabilize the OM of E. coli was monitored by the NPN assay (Fig. 6B.5). It is evident that the bacteria do disrupt the OM structure to facilitate the influx of NPN. The effect of divalent cations, MgCl₂ and CaCl₂ on the antibacterial activity of these peptides was examined. Divalent cations stabilize the OM and are known to inhibit the activity of host-defense peptides. The data shown in Fig. 6B.6 indicate that the effects of divalent cations are variable. At 0.25 mM Mg²⁺ and Ca²⁺ concentrations, there is only partial inhibition of activity of PH1 and PH3, whereas, PH2 is completely inhibited at this concentration. At 2 mM Ca²⁺ or Mg²⁺, the activity of all the three peptides was inhibited.

6B.3.5 Inner membrane permeabilization: The ability of the peptides to permeabilize the inner membrane of E. coli was examined with ONPG influx (Fig. 6B.7). Permeabilization of the bacterial cytoplasmic membranes was slow compared to a membrane lytic, α-helical peptide 27P. Effective permeabilization is observed with PH1 and PH2, while PH3 did not show permeabilization.

Membrane potential sensitive cyanine dye has been used to detect membrane permeabilization changes in the bacterial inner membrane in the presence of NaCl. Changes in the fluorescence of cyanine dye, when peptides were added to E. coli DC2 cells (LC against E. coli DC2 was similar to E. coli 160.37 and E. coli MG 1655), are
Fig. 6B.3: Effect of peptides on *E. coli* W160.37 visualized by transmission electron microscopy. (A) *E. coli* control, (Magnification 20,000). Bacterial cells incubated with (B) with PH1 (Magnification 33,000) (C) with PH2 (Magnification 20,000) (D) with PH3 (Magnification 33,000). Bacteria incubated with 50% lethal concentration. Arrows indicate contents leaking out of the cell.
Fig. 6B.4: CD spectra of human beta defensins.

(A) PHI in buffer, SDS (B) PHI in TFE (C) PH2 in buffer, SDS (D) PH2 in TFE (E) PH3 in buffer, SDS (F) PH3 in TFE.

Key. (○) Buffer, (●) SDS and (■) TFE
Fig. 6B.5: Effect of human β-defensin analogs on the outer membrane permeability in *E. coli* measured as an increase in the fluorescence intensity of (NPN), Ex. 350 nm, Em. 420 nm.

(A) PH1 (B) PH2 (C) PH3.

Key: C-control;

(1) 1 μM; (2) 3 μM; (3) 6 μM.
Fig. 6B.6: Effect of divalent cations on the antibacterial activity of human β-defensin analogs. (A) MgSO₄ (B) CaCl₂. Concentration of divalent cations:
Open bars (0.25 mM), Dark bars (2 mM)
Fig. 6B.7: Kinetics of hydrolysis of ONPG due to inner membrane permeabilization in presence of human β-defensin analogs was monitored at 420 nm.

Key: Concentration of peptides PH1, PH2, PH3 used are 10 μM (■), 20 μM (●), 30 μM (▲). Concentration of 27P used: 4 μM (■), 7 μM (●), 14 μM (▲).
shown in Fig. 6B.8. All the three peptides cause changes in cyanine dye fluorescence to different extents in the presence of varying NaCl concentrations. This shows that the peptide have the ability to modulate IM permeabilization in the presence of salt.

6B.3.6 Effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP) and glucose on antibacterial activity and salt inactivation: The effect of depolarization of bacterial inner membrane potential for antibacterial activity was examined using CCCP. The data are presented in Fig. 6B.9. It appears that salt sensitive peptide analogs in the presence of CCCP or CCCP with only either glucose or NaCl have antibacterial activity. Thus peptides regained activity when CCCP was included with NaCl. But their activity was lost when glucose was included with CCCP and NaCl (data not shown). Hence, salt inhibition is also influenced by membrane potential in addition to electrostatic interactions.

6B.4 Discussion

Human defensins are of significant interest due to their broad-spectrum antimicrobial activities and chemotactic activities towards T-lymphocytes and immature dendritic cells (Chertov et al., 1996; Yang et al., 1999; Yang et al., 2000). Each of the human defensins has a specific biological role as far as antibacterial activity and salt inactivation properties are concerned in the innate immune system (Singh et al., 1988; Harder et al., 1997; Harder et al., 2001). HBD-1 is constitutively expressed with specificity for Gram-negative bacteria, whereas the expression of HBD-2 and HBD-3 is constitutive and inducible, exhibiting broad microbicidal activity (Schibli et al., 2002). Although the three defensins, HBD-1, HBD-2 and HBD-3 possess sequence similarities, their properties are relatively distinct. HBD-3 has approximately twice the net positive charge density (+11) compared to HBD1 (+5) and HBD2 (+6) (Schibli et al., 2002). Several mechanisms of antibacterial activity of defensins have been postulated. They involve pore formation (Wimley et al., 1994), local surface oligomerization (Cocianich et al., 1993) and voltage-dependent ion gating (Kagan et al., 1990). The specificity of defensin interactions with microbial membranes and inactivation at elevated ionic
Fig. 6B.8: Kinetics of cyanine dye fluorescence intensity changes with *E. coli* DC2 cells in presence of different concentrations of NaCl with 10 μM human β-defensin analogs. (A) PH1 (B) PH2 (C) PH3
Fig. 6B.9: Effect of CCCP and NaCl on antibacterial activity of human beta defensin analogs (A) PH1 (B) PH2 (C) PH3 in the presence of 100 mM NaCl. Open bars represent *E. coli* and dark bars represent *S. aureus.*
strength is not well understood. In order to understand the antibacterial activity and salt sensitivity it is necessary to design defensin analogs based on requirements of charge, amphiphilicity, structure and number of disulphide bonds. This involves synthesis and structure-function studies. We have synthesized the C-terminal single disulphide analogs of HBD1-3 to understand the sequence requirement for specificity for bacteria and mechanism of salt inactivation.

We observe that the synthetic peptides corresponding to the C-terminal fragments of HBD-1 to 3 i.e. PH1, PH2 and PH3 possess antibacterial activity against Gram-negative as well as Gram-positive bacteria, and salt inactivation of antibacterial activity is dependent on the specific bacteria and concentration of NaCl used. However, the specificity of the analogs for an organism and the salt-inactivation properties are different compared to the parent peptides. So it is possible that the N-terminal regions also contribute to the antibacterial activities of the parent peptides although the cationic residues are clustered at the C-terminus. Recently, with the help of synthetic analogs the significant role of primary sequences for antibacterial activity and chemotactic activity of HBD-3 was achieved through disulphide engineering. It has been shown that the antibacterial activity of HBD-3 is independent of its pattern of disulphide pairings. The replacement of cysteine residues with α-amino butyric acid rendered the peptide chemotactically inactive with enhanced antibacterial activity. The lack of chemotactic activity was attributed to lack of native disulphide folding of the peptide (Wu et al., 2003). The studies on short analogs of HBD-3, replacing cysteines with α-aminobutyric acid, showed antibacterial activities with altered specificities towards bacteria (Hoover et al., 2003).

The overlapping CD spectra of the peptides in buffer and SDS micelles indicate that there are no significant changes in the secondary structure of peptide analogs. This shows that peptides are already structured in aqueous buffer and addition of membrane-mimetic micelles did not have an effect on secondary structure. Similar results were observed with HBD-2 and its analog with aspartic acid deletion (Antcheva et al., 2004). But in TFE, the spectra were found to be different, compared to buffer and SDS spectra. The difference in TFE spectra stresses the significance of microenvironment and the
presence of different conformer populations of these peptides in different solvent systems (Ladokhin et al., 1999).

The outer membrane permeabilization of bacteria by the analogs has been confirmed by monitoring the NPN fluorescence changes. Permeabilization of the cytoplasmic membranes of *E. coli* was evaluated by following the unmasking of cytoplasmic β-galactosidase to the extracellular chromogenic substrate, O-nitrophenylgalactoside (ONPG), and also by measuring the disruption of the electrical potential gradient across the cytoplasmic membrane of intact bacteria. The utilization of measurement of normally impermeable substrate to cytoplasmic β-galactosidase in IM permeabilization assays suffer from using a bulky substrate (O-nitrophenyl galactoside) as well as an inability to fully dissociate cytoplasmic membrane from outer membrane permeabilization (Wu et al., 2003; Wu et al., 1999a). The kinetics of ONPG release in permeabilization of cytoplasmic membrane was slow compared to any α-helical peptide. ONPG release was not seen in presence of PH3 whereas, it showed enhancement in the fluorescence intensity of the membrane potential-sensitive cyanine dye, which is an indication of permeabilization of IM. The increase in fluorescence of cyanine dye is more in the presence of 20 mM NaCl compared to 100 mM NaCl, where the membrane potential is maximum. Thus the IM permeabilization is proton motive force-dependent and alters the permeability of the cells in the presence of salt.

Electrostatic interactions between peptides and bacterial surfaces are crucial in the activity of cationic, antimicrobial peptides such as defensins. The divalent cations and mitochondrial metabolism affected the candidacidal activity of HNP defensins (Lehrer et al., 1988). It has been proposed that peptides that form pores or channels are inserted into membranes in a voltage-dependent manner. The insertion is facilitated by membrane potential. Therefore, these peptides displayed reduced bactericidal effect on bacteria pretreated with an uncoupler that discharges pH gradient and destroys the proton motive force (Lehrer et al., 1988). The activities of human defensin analogs, PH1, PH2 and PH3 are affected by the divalent cations and NaCl. The peptides kill bacteria effectively even when the IM is depolarized by proton conductor CCCP. These observations suggest that
electrostatic interactions affect the antibacterial activity of human β-defensin analogs, which also explains the salt inactivation of these peptides. No inhibition of activity of peptide analogs was observed when CCCP was included with NaCl. When the membrane depolarizing effect of CCCP was reversed by the addition of an energy source such as glucose, reversal in the activity was observed (i.e. peptide analogs became inactive again). This observation suggests that proton motive force also plays a crucial role in salt inactivation of these peptides in addition to electrostatic interactions.

PH1, PH2 and PH3 have a cluster of positive charges at the C-terminal end. The above data indicate that the mechanism of inhibition of bacterial growth does not depend on proton motive force. The primary event is electrostatic interactions between the positive charges of the peptide and anionic sites on the membrane followed by membrane permeabilization. As reported earlier, a correlation between membrane permeability and antibacterial activity does not always exist for many β-sheet antibiotic peptides (Sitaram and Nagaraj, 1999; Shai, 1999; Wu et al., 1999b; Zhang et al., 2001). Salt effect is due to modulation of membrane potential. At high external salt, IM potential is altered due to which activity is abolished. Peptides showed activity in the presence of salt when membrane potential was dissipated with CCCP. Mammalian defensins share the same topology of a β-sheet cross-linked by three disulphide bonds whereas only β-defensins contain α-helix at the N-terminus. There are only minor differences in the tertiary structures of the three human β-defensins. Shorter peptides derived from native sequence help in elucidating the mechanisms and specificity of these peptides. The activity profiles exhibited by these analogs give information about significance of their structure with function. This may help in designing a novel class of small molecule antibiotics with broad specificity for therapeutic applications.

6B.5 Summary

- C-terminal fragments of HBD-1, HBD-2 and HBD-3, (PH1, PH2 and PH3 respectively), were chosen for investigations to understand their antibacterial activities and mechanism of action.
• The peptides showed antibacterial activities, which was lost in the presence of salt. The minimal threshold concentrations of NaCl required for inactivation of these peptides against *E. coli* and *S. aureus* was found to be different for each of these peptides. The salt inactivation properties of PH3 analog on *S. aureus* are different compared to parent peptide.

• The peptides showed different activity spectrum at lower concentration of divalent cations such as Ca$^{2+}$ and Mg$^{2+}$ and lost their activity at high concentration.

• The conformational studies as studied by CD spectroscopy show that the peptides have ordered conformation both in aqueous environment and in SDS micelles.

• The peptides permeabilized the outer membrane.

• The inner membrane permeabilization was studied with ONPG influx. The kinetics of hydrolysis of ONPG was different compared to the α-helical peptide 27P. The PH3 analog did not show ONP release, whereas increase in fluorescence of cyanine-dye was observed which is an indication for permeabilization of cytoplasmic membrane.

• Depolarization of bacterial cell membrane potential with CCCP resulted in antibacterial activity in the presence of NaCl.

• These studies indicate that C-terminal fragments have antibacterial activity. Modulation of proton motive force of the cell in the presence of high NaCl is probably the reason for salt inactivation in addition to electrostatic interaction with the bacterial inner membrane.

Studies reported in this Chapter and by others indicate that the conserved spacings and disulphide connectivities in β-defensins is not essential for exhibiting antibacterial activities. The order of disulphide bonds in defensins could be a biosynthetic requirement and possibly for interacting with the cellular components of adaptive immunity. Since
linear and a single disulphide bond defensin analogs do appear to interact with mast cells, it would be of interest to examine in detail, the structural requirements in defensins for recruiting components of adaptive immunity at infection sites. The C-terminal segment, which has most of the cationic residues, emerges as an important determinant of antibacterial activity. The observation that, short peptides related to defensins with a single disulphide or even without disulphide bonds can have antibacterial activity, make them attractive candidates for development as therapeutic agents to treat bacterial infections in humans and possibly other mammals.