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Antimicrobial activity of human β-defensin 4 analogs: Insights into the role of disulfide linkages in modulating activity

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ABSTRACT

Human β-defensins (HBDs) are cationic antimicrobial peptides that are components of the innate immune system. They are characterized by three disulfide bridges. However, the number of cationic residues as well as the presence of lysine and arginine residues vary. In HBD4, the cationic residues occur predominantly in the N-terminal segment, unlike in HBD1–3. We have examined the antimicrobial activity of peptides spanning the N- and C-terminal segments of HBD4. We have introduced one, two and three disulfide bridges in the peptides corresponding to the N-terminal segments. Peptides corresponding to the N-terminal segment had identical sequences and variation was only in the number and spacing of cysteines and disulfide bridges. Antimicrobial activity to varying extents was observed for all the peptides. When two disulfide bridges were present, decrease in antimicrobial potency as well as sensitivity of activity to salt was observed. Enhanced antimicrobial activity was observed when three disulfide bridges were present. The antimicrobial potency was similar to HBD4 except against *Escherichia coli* and was attenuated in the presence of salt. While the presence of three disulfide bridges did not constrain the peptide to a rigid β-sheet, the activity was considerably more as compared to the peptides with one or two disulfide bridges. The peptides enter bacterial and fungal cells rapidly without membrane permeabilization and appear to exert their activity inside the cells rather than at the membrane.

1. Introduction

The cationic antimicrobial peptides defensins are important components of innate immunity [13,14,30,46]. Defensins have been the subject of extensive investigations with a view to understand how these peptides exert their antimicrobial activity [2,5,11,15,17,36]. They are classified into α, β and θ defensins on the basis of disulfide connectivity. Human β-defensins (HBDs) are expressed predominantly in mucosal and epithelial surfaces [13,24,36]. Disulfide connectivities in β-defensins are between cysteines 1, 5; 2, 4 and 3, 6 [13,31,36,42]. Structural studies on HBD1–3 have indicated that the β-strands occurring at the middle and C-terminal regions are conserved [3,19,20,26,40,41].

Bactericidal and fungicidal activities of these peptides vary considerably and are attenuated at high ionic strength [36]. HBD1 and 2 kill Gram-negative bacteria and fungus more efficiently as compared to Gram-positive bacteria at low ionic strength. HBD3 is more potent against Gram-positive and Gram-negative bacteria as compared to HBD1, 2 and the activity is not attenuated at high salt concentrations [2,16,17,28,51]. HBD4 exhibits broad spectrum antimicrobial activity but only at low ionic strength [15]. The antimicrobial activities of smaller fragments of β-defensins, with or without disulfide constraints, have been investigated for a better understanding of structure–activity relationships [21,27–29,44]. Recently, it has been demonstrated that HBD analogs composed of different regions of HBD1 and 3 possess enhanced antibacterial activity at high salt concentrations and internal regions of HBD1 and C-terminal region of HBD3 are essential for tolerance to salt [44]. Chimeras of HBD2 and 3 have been recently shown to have potent activity that is not attenuated in the presence of high salt concentrations [23]. Linear analogs of HBD3 also show bactericidal activity comparable to the native peptide [8,58]. Reduced HBD1 shows potent antimicrobial activity against *Candida albicans* and anaerobic Gram-positive species *Bifidobacterium* and *Lactobacillus* [43]. We have previously shown that C-terminal analogs of HBD1–3 with a single disulfide bridge kill bacteria and fungi [28,29].

Although the cysteine connectivity in HBD4 is identical to other β-defensins, the distribution of cationic residues in the primary sequence is different. Cationic residues in HBD1–3 occur predominantly at the C-terminal end and after the third cysteine residue [13,36,49], while in HBD4, 6 of the 10 cationic residues are localized between residues 1 and 18 [15]. Also, 5 of the 6 cationic residues in this N-terminal segment occur sequentially, interspaced by cysteine residues as RCRKKCR. Therefore, it would be of interest to delineate the determinants of antimicrobial activity in HBD4.

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Table 1
Primary structures of HBD1–4 and net charge at neutral pH.

Peptide	Sequence[a]	Net charge
HBD1	DHYN**C**^{1}VSSGGQ**C**^{2}LYSA**C**^{3}PIFTKIQGT**C**^{2}YRGKAK**C**^{1}**C**^{3}K	+4
HBD2	GIGDPVT**C**^{1}LKSGAI**C**^{2}HPVF**C**^{3}PRRYKQIGT**C**^{2}GLPGTK**C**^{1}**C**^{3}KKP	+6
HBD3	GIINTLQKYY**C**^{1}RVRGGR**C**^{2}AVLS**C**^{3}LPKEEQIGK**C**^{2}STRGRK**C**^{1}**C**^{3}RRKK	+11
HBD4	ELDRI**C**^{1}GYGTAR**C**^{2}RKK**C**^{3}RSQEYRIGR**C**^{2}PNTYA**C**^{1}**C**^{3}LRK	+7
N1	ELDRI**C**^{1}GYGTARRKK**C**^{1}R	+4
N2	ELDRIGYGTAR**C**^{1}RKK**C**^{1}R	+4
N3	ELDRI**C**^{1}GYGTAR**C**^{1}RKKR	+4
N4	EL**C**^{1}DRI**C**^{2}GYGTAR**C**^{1}RKK**C**^{2}R	+4
N5	EL**C**^{1}**C**^{2}DRIG**C**^{3}YTAR**C**^{2}RKKRS**C**^{1}**C**^{3}L	+4
C1	KRSQEYRIGR**C**^{1}PNTYA**C**^{1}LKR	+5

[a] Disulfide connectivities are shown by superscript numbers adjacent to cysteines. Cationic residues are underlined.

Synthetic peptides were designed spanning N and C-terminal regions of the sequence with disulfide bridges (Table 1). We have observed that peptides spanning the N- and C-terminal regions of HBD4 exhibited antimicrobial activity. The presence of three disulfide bridges in the N-terminal segment resulted in enhanced antimicrobial activity against *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *C. albicans*. A peptide corresponding to the C-terminal segment was active against only *E. coli* and *C. albicans*. The peptides appear to kill bacteria and fungi not by permeabilizing membranes but by rapidly crossing the membrane barrier and exerting their activity inside the cells.

2. Materials and methods

2.1. Reagents

9-Fluorenylmethoxy carbonyl (F-moc) amino acids were obtained from Novabiochem AG (Switzerland) and Advanced Chemtech (Louisville, KY). N-Fmoc-N′-(4-methoxy-2,3,6-trimethylbenzenesulfonyl)-L-arginine polyethyleneglycol-polystyrene (F-moc-L-Arg(Mtr)-PEG-PS) and polyethyleneglycol –polystyrene (PEG–PS) were purchased from Millipore (Bedford, MA). N-hydroxybenzotriazole hydrate (HOBt) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Advanced Chemtech (Louisville, KY). Phospholipids PC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), PE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), and PG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol)) (sodium salt), were purchased from Avanti Polar Lipids (Alabaster, AL). CF (carboxyfluorescein) was obtained from Sigma–Aldrich, India. N-(3-triethylammoniumpropyl)-4-(6-(4(diethylamino) phenylhexatrienyl) pyridinium dibromide) (FM4-64), and propidium iodide (PI), were obtained from Molecular Probes. HBD4 (Code: 4406-s, Lot No. 590707) was purchased from Peptide Institute Inc. (Osaka). All other chemicals used were of the highest grade available.

2.2. Peptide synthesis

HBD4 analogs N1–N5 and C1 (Table 1) were synthesized using Fmoc chemistry [1]. F-moc-L-Arg(Mtr)-PEG-PS (0.13 mmol/g) and PEG–PS resin (0.19 mmol/g) were used as solid support. Peptides were cleaved from the resin using a mixture containing 80% trifluoroacetic acid (TFA), 8% m-cresol, 8% thioanisole, and 4% ethanedithiol for 12–15 h at room temperature. Peptides were precipitated on ice-cold diethyl ether. Disulfide bond formation was accomplished in peptides having two cysteine residues by carrying out oxidation at a concentration of 0.4 mg/ml in 20% dimethylsulfoxide (DMSO) for 12–14 h [48]. Purification of peptides was carried out on a Hewlett Packard 1100 series HPLC instrument using a reverse-phase Agilent 300SB-C18 Zorbax column with a water (solvent A) and acetonitrile (solvent B) containing 0.1% TFA. Peptides were eluted using a linear gradient from 5% to 100% of solvent B over 60 min at a flow rate of 0.5 ml/min.

Two and three disulfide bridged peptides N4 and N5 were synthesized using orthogonal protecting group for side chain of cysteines. In the two disulfide containing peptide, N4, 1st and 3rd cysteines were protected with acetoamidomethyl (Acm) group and 2nd and 4th cysteines were protected with trityl group (Trt). In the three disulfide containing peptide, N5, 1st and 5th cysteines were protected with Acm, 2nd and 4th cysteines were protected with tertiary butyl (t-Bu) and 3rd and 6th cysteines were protected with Trt group which was removed during the acidic cleavage of peptide from resin. The first disulfide bond was formed in 20% DMSO [48]. Peptide was purified by reverse phase chromatography and was subjected for second disulfide formation by deprotecting Acm group. It was removed by iodine oxidation as mentioned elsewhere [27]. In brief, peptide was dissolved in acetic acid:water (4:1) at the concentration of 50–100 μg/ml and 20 equivalents of iodine were added into peptide. Solution was kept for 6–8 h at 37 °C. Reaction was stopped by adding two times volume of deionized water. Iodine was removed from the mixture by washing with excess carbon tetrachloride until solution was colorless. The peptide was then purified by HPLC. The 3rd disulfide was formed by removing the t-Bu group by the method described by Kluver et al. [27] with few modifications. The peptide was dissolved in TFA (50 μg/ml). Reaction mix was formed by adding 500 equivalent of DMSO and 100 equivalent of anisole to the peptide solution. Mix was stirred overnight as it was found that incomplete oxidation occurs in 2 h of incubation. The peptide was then purified by reverse phase chromatography using linear gradient of 5–100% solvent B (acetonitrile containing 0.1% TFA, v/v) in 60 min on reverse phase C-18 column (Agilent 300 SB C-18 Zorbax). All the peptides were characterized by mass spectrometry on an AB4800 matrix assisted laser desorption ionization-time of flight/time of flight (MALDI TOF/TOF) mass spectrometer from Applied Biosystems (PerSeptive Biosystems, Foster City, CA). Peptides with two or three disulfide linkage were purified after each oxidation step and then were characterized by MALDI TOF/TOF before keeping for next oxidation step.

For CF labeling, peptide attached to the resin was incubated with a mixture containing CF and activating agent (HOBt and HBTU) in DMF [56] and labeled peptides were cleaved from resin as described above. All the purified peptides were dried and dissolved in deionized water. The concentrations of peptides were determined using a molar absorption coefficient of 1280 M^{-1} cm^{-1} at 280 nm and for CF labeled peptide at 492 nm using coefficient of 65000 M^{-1} cm^{-1}. The concentration was also cross-checked by dissolving known weight of dried peptide in deionized water.