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Structure and Antibiotic Activity of a Porcine Myeloid Antibacterial Peptide, PMAP-23 and its Analogues

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PMAP-23 is a 23-residue antimicrobial peptide derived from porcine myloid cells. In order to investigate the effects of two Pro residues at positions 12 and 15 of PMAP-23 on antibiotic activity, two analogues in which Ala was substituted for Pro residue at position 12 or 15 were synthesized. Pro^{12}→Ala (PMAP1) or Pro^{15}→Ala (PMAP2) substitution in PMAP-23 caused a significant reduction on antitumor and phospholipid vesicle-disrupting activities, but did not cause a significant effect on antibacterial activity. PMAP-23 displayed the type I β-turn structure with a negative ellipticity at near 205 nm in SDS micelle, whereas PMAP1 and PMAP2 had a somewhat α-helical propensity in TFE solution, as compared to PMAP-23. These results suggest that two Pro residues of positions 12 and 15 in PMAP-23 play important roles in the formation of β-turn structure on lipid membrane and its β-turn structure may be essential for antibiotic activity including phospholipid vesicle-disrupting property.

Keywords: Antibacterial activity, Antitumor activity, Phospholipid vesicle-disrupting activity, PMAP-23, Secondary structure.

Introdution

Leukocytes are important elements in the host defense against microbial infections. The microbicidal action of leukocytes emanates through the oxidative and non-oxidative mechanism. The non-oxidative mechanism of leukocytes are mediated from the antimicrobial peptides stored within its various cytoplasmic granules. A variety of antimicrobial peptides named as the cathelicidin family have been identified from leukocytes (Gudmundsson *et al.*, 1996; Gallo *et al.*, 1997; Ganz and Lehrer., 1997; Panyutich *et al.*, 1997). Members of the cathelicidin family have a conserved N-terminal cathelin domain of about 100 residues and a C-terminal antimicrobial domain of varied length. According to common structural features, these antimicrobial peptides are grouped into amphipathic α-helical (PMAP-36, PMAP-37, CRAMP and LL-37) (Bagella *et al.*, 1995; Mahoney *et al.*, 1995; Tossi *et al.*, 1995; Skerlavaj *et al.*, 1996), Cys-rich (protegrins) (Zhao *et al.*, 1994), Pro- and Arg-rich (Bac5, Bac7 and PR-39) (Storici and Zanetti, 1993; Zanetti *et al.*, 1993), and Trp-rich peptides (indolicidin) (Del Sal *et al.*, 1992).

The sequence of a novel cathelicidin family from cDNA of porcine myeloid mRNA was reported (Zanetti *et al.*, 1994). The predicted polypeptide includes a prepropeptide of 153 residues and a putative antimicrobial domain of 23 residues in its C-terminus. The peptide of 23 amino acid residues corresponding to this antimicrobial domain was termed porcine myeloid antibacterial peptide-23(PMAP-23) (Zanetti *et al.*, 1994). PMAP-23 contains 7 positive charged amino acids (5 Arg and 2 Lys) and 11 hydrophobic residues (2 Ile, 2 Leu, 1 Phe, 2 Trp, and 4 Val). Accordingly, PMAP-23 is expected to have an amphipathic α-helical structure which is typical of antibacterial peptides including cecropins and magainins. However, two Pro residues in the central region of PMAP-23 precludes α-helical conformation but induces β-turn structure.

In the present study, to investigate the effects of two Pro residues of PMAP-23 on antibiotic activities containing antibacterial, antitumor and membrane disruption activity, PMAP-23 analogues (PMAP1 and PMAP2) with Ala-substitution for Pro at positions 12 or 15 were synthesized. Lytic activities of the peptides against Gram-positive bacteria, Gram-negative bacteria, tumor cells, and phospholipid-vesicle were measured. Also, in order to investigate the structure-antibiotic actvity relationships of PMAP-23, the secondary structure of the peptides in the cell membrane-mimicking environment such as TFE or SDS micelle was measured using CD spectra.

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Materials and Methods

Peptide synthesis The peptides were synthesized by the solid phase method using Fmoc(9-fluorenyl-methoxycarbonyl)-chemistry (Merrifield, 1986; Shin *et al.*, 1996, 1997, 1999). Arg(pmc)-Wang-Resin was used as the support to obtain a C-terminal free peptide. The coupling of Fmoc-amino acids was performed with N-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC). Amino acid side chains were protected as follows: tert-butyl (Asp and Thr), trityl (Gln), tert-butyloxycarbonyl (Lys and Trp), pmc (Arg). Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1, 2-ethandithiol and triisopropylsilane (82.5:5:5:5:2.5:2, v/v) for 3 hr at room temperature. The crude peptide was then repeatedly washed with diethylether, and dried in a vacuum. The crude peptides were purified by a reversed-phase preparative HPLC on a Waters 15-μm Deltapak C_{18} column (19×30 cm). Purity of the purified peptides was checked by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (Beckman, San Ramon, USA), 4.6×25 cm. The purified peptides were hydrolyzed with 6 N-HCl at 110 °C for 22 h, and then dried in a vacuum. The residues were dissolved in 0.02 N-HCl and subjected to an amino acid analyzer (Hitachi Model, 8500 A, Tokyo, Japan). Peptide concentration was determined by amino acid analysis. The molecular masses of the peptides were confirmed with MALDI (matrix-assisted laser desorption/ionization) mass spectrometer.

Hemolytic activity The hemolytic activity of the peptides was evaluated by determining hemoglobin release of the suspensions of fresh human erythrocytes at 414 nm (Andreu *et al.*, 1992; Shin *et al.*, 1996, 1997, 1999). Human red blood cells were centrifuged and washed three times with phosphate-buffered saline (PBS: 35 mM phosphate buffer/0.15 M NaCl, pH 7.0). One hundred μl of human red blood cells suspended 8% (v/v) in PBS were plated into 96-well plates, and then 100 μl of the peptide solution was added to each well. The plates were incubated for 1 h at 37°C, and centrifuged at 150 x g for 5 min. One hundred μl aliquots of the supernatant were transferred to 96-well plates. Hemolysis was measured by absorbance at 414 nm with an ELISA plate reader (Molecular Devices Emax, Sunnyvale, USA). Zero percent hemolysis and 100% hemolysis were determined in PBS and 0.1% Triton-X 100, respectively. The hemolysis percentage was calculated using the following equation: % hemolysis = [($Abs_{414\ nm}$ in the peptide solution-$Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton-X 100-$Abs_{414\ nm}$ in PBS)]×100.

Antibacterial activity *Escherichia coli* (KCTC 1682), *Salmonella typhimurium* (KCTC 1926), *Pseudomonas aeruginosa* (KCTC 1637), *Bacillus subtilis* (KCTC 1918), *Streptococcus pyogenes* (KCTC 3096), and *Staphylococcus. aureus* (KCTC 1621) were supplied from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (Taejon, Korea). The bacteria were grown to the mid-logarithmic phase in a medium (g/l) [10 bactotryptone/5 yeast extract/10 NaCl (pH 7.0)]. The peptides were filtrated through a 0.22 μm filter and stepwise-diluted in a medium of 1% bactopeptone. The tested organism (final bacterial suspension: 2 $\times 10^6$ colony formation units (CFU)/ml) suspended in growth medium (100 μl) was mixed with 100 μl of the two-fold diluted serial solution of each peptide in a microtiter plate well with three replicates for each test sample. The plates were incubated for 18 h at 37°C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide which gave no visible growth on the plate.

Antitumor activity Antitumor activity of the peptides against tumor cells was determined as 50% inhibition concentration (IC_{50}) using a tetrazolium (MTT) calorimetric assay (Shin *et al.*, 1999). Human chronic myelogenous leukemia (K-562: ATCC cat no. CCL-243), human acute T cell leukemia (Jurkat: ATCC cat no. TIB-152), human lung carcinoma cancer cells (A-549: ATCC cat no. CCL-185) and human breast adenocarcinoma cell (MDA-MB-361: ATCC cat no. HTB-27) were used for the growth inhibitory activity assay of the peptides against tumor cells. These cells were obtained from the Genetic Resources Center of Korea Research Institute of Bioscience & Biotechnology (Taejon, Korea). The cells were grown in a RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. The cells were plated on 96 well plates at a density of 2.0×10^4 cells/well in 150 μl of the same medium. After incubating the plates overnight at 37°C in 5% CO_2 atmosphere, 20 μl of serially diluted peptides were added and then incubated for 3 days. Twenty μl of tetrazolium (MTT) solution (5 mg/ml MTT in phosphate-buffered saline) was added to each well and the plates were incubated at 37°C for 4 h. Forty μl of 20% SDS solution containing 0.02 M-HCl was added to dissolve the dark blue crystals which formed (MTT-formazan product) in each well, and then incubated overnight. Absorbance was measured at 570 nm on an ELISA plate reader (Molecular Devices Emax, Sunnyvale, USA).

Carboxyfluorescence-leakage experiment Carboxyfluorescein (CF)-encapsulated large unilamellar vesicles (LUV) composed of PC/PS (4:1, w/w) were prepared by the reversed-phase ether evaporation method (Duzgunes *et al.*, 1983) using 100mM CF. The initially formed vesicles were extruded through Nuleopore filter of 0.1μm. To remove free CF dye, the vesicles were passed through a Bio-Gel A 0.5m (Bio-Rad, Richmond, USA) column (1.5×30cm) using phosphate buffered saline, pH 7.4, as the eluting buffer. The separated LUV fraction, after appropriate dilution to a final concentration of 6.36 μM, was mixed with the peptide solution in a 2 ml quartz cuvette at 25°C. The leakage of CF from the LUV was monitored by measuring fluorescence intensity at 520 nm excited at 490 nm on a Shimazu RF-5000 spectrofluorometer (Tokyo, Japan). The percentage of dye-release caused by each peptides was calculated by the equation 100×(F-F_0) / (F_t-F_0) where F_0 is the initial fluorescence intensity, F_t total fluorescence intensity observed after addition of 20 μl of 10% Triton X-100, and F fluorescence intensity in the presence of peptide.

CD measurement The CD measurement of the peptides were made on a Jasco J720 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) using a cell of 1 mm pathlength. The CD spectra of

the peptides in 10 mM sodium phosphate buffer (pH 7.2), 50% (v/v) TFE or 30 mM SDS containing 10 mM sodium phosphate buffer (pH 7.2) were recorded at 25°C in the 190-240 nm wavelength range at 0.1 nm intervals. The peptide concentrations were 100 μM. For each spectrum, the data of four scans was averaged and smoothed by the J 720/98 system program (ver. 120C). CD data were expressed as mean residue ellipticity [θ]. The mean residue ellipticity [θ] was calculated using the molecular weight of each peptide as determined from the amino acid composition (Chen *et al.*, 1974).

Results

Peptide synthesis All peptides used in this study were prepared by the solid phase technique using Fmoc(9-fluorenylmethoxycarbonyl)-chemistry. The crude peptides were obtained from TFA treatment in the presence of scavengers and purified by the reverse-phase HPLC. The primary structure of the synthetic peptides was summarized in Table 1. All synthetic peptides had the expected amino acid composition by amino acid analysis (data not shown). The correct molecular weights of the synthetic peptides were confirmed by MALDI mass spectrometry (Table 2).

Table 1. Amino acid sequence of PMAP-23 and its analogues with Ala-substitution

Peptides	Sequences
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR
PMAP1	RIIDLLWRVRRAQKPKFVTVWVR
PMAP2	RIIDLLWRVRRPQKAKFVTVWVR

The bolded amino acids indicate substituted amino acid in PMAP-23.

Table 2. Molecular weight of PMAP-23 and its analogues determined by MALDI-MS.

Peptides	Observed value	Calculated value
PMAP-23	2962.6	2963.4
PMAP1	2934.7	2933.4
PMAP2	2936.6	2936.8

Antibacterial, antitumor and hemolytic activity Antibacterial activities of the peptides for gram-positive and gram-negative bacteria were determined as minimal inhibitory concentration (MIC) by the microdilution test (Shin *et al.*, 1996, 1997, 1999; Kim *et al.*, 1999) (Table 3). PMAP-23 showed potent antibacterial activity against both gram-negative bacteria (*E. coli,* and *S. typhimurium*) and gram-positive bacteria (*B. subtilis* and *S. pyogenes*) at concentration ranging from 3.125 to 6.25 μM. In *P. aeruginosa* and *S. aureus,* PMAP-23 displayed lower lytic activity (MIC: 12.5 μM) rather than other bacterial strains. $Pro^{12} \rightarrow$ Ala (PMAP1) and $Pro^{15} \rightarrow$ Ala (PMAP2) substitution in PMAP-23 did not affect on antibacterial activity. Antitumor activity of the peptides was determined as 50% inhibition concentration (IC_{50}) by the MTT colorimetric assay. K-562, Jurkat, A-549 and MDA-MB-361 tumor cells were used for the antitumor activity of the peptides. As shown in Table 4, PAMP1 exhibited 2-fold lower activity in antitumor activity than PMAP-23. In contrast, IC_{50} of PMAP2 was almost inactive at 100 μM. The hemolytic activity as the cytotoxicity for the peptides was evaluated by determining hemoglobin release of the suspensions of fresh human erythrocytes at 414 nm (Table 4). PMAP-23 was unable to lyse human erythrocytes even at 100 μM.

Phospholipid vesicle-disrupting activity In order to investigate the membrane-interacting mechanism of the peptides, the vesicle perturbation activities induced by the peptides were measured using the CF-entrapped acidic phospholipid vesicle composed of PC/PS (4:1, w/w) as the artificial model lipid membrane. As shown in Fig. 1, the rank order of the vesicle-disrupting activity of the peptides was PMAP-23 > PMAP1 > PMAP2. These peptides induced the

Table 3. Antibacterial activities of PMAP-23 and its analogues with Ala-substitution.

Peptides	MIC : μM					
	E. coli	*S. typhimurium*	*P. aeruginosa*	*B. subtilis*	*S. pyogenes*	*S. aureus*
PMAP-23	3.1	3.1	12.5	6.3	3.1	12.5
PAMP1	6.3	3.1	6.3	3.1	1.6	12.5
PMAP2	6.3	3.1	12.5	6.3	3.1	6.3

Table 4. Antitumor and hemolytic activities of PMAP-23 and its analogues with Ala substitution.

Peptides	IC_{50} : μM				% Hemolysis (100 μM)
	MDA-MB-361	Jurkat	A-549	K-562	Human erythrocyte
PMAP-23	25	30	25	30	0
PMAP1	50	75	50	50	0
PMAP2	>100	>100	>100	>100	0

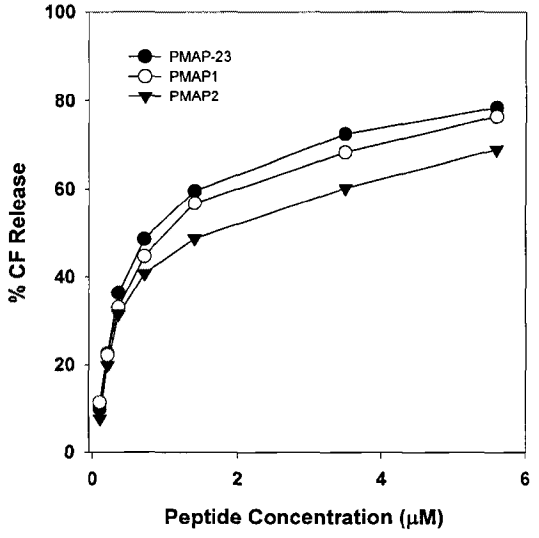

Fig. 1. Release of CF from LUVs composed of PC : PS (4 : 1). The extents of vesicle disruption induced by peptide were indicated as a function of peptide to lipid ratio (P/L). The released CF fluorescence was measured at ex. = 490 nm and em. = 520 nm.

release of CF from phospholipid vesicle in a dose dependent manner. The relative efficiency of the peptides to perturb the lipid membranes correlated to their antitumor activities. This result suggests that the antitumor activity induced by the peptides involves in the interaction of cell membrane with the peptide, and subsequent membrane perturbation resulting in cell death.

Structure analysis The CD spectra of the peptides were measured in TFE solution as α-helix promoting solvent or sodium dodecyl sulfate (SDS) solution as lipid membrane-mimicking solvent (Fig. 2). In 10 mM sodium phosphate buffer, PMAP-23 and its analogues showed an unordered structure, which had a strong negative ellipticity at near 200 nm. PMAP-23 and its analogues gave a negative ellipticity at near 205 nm of a typical pattern seen in the type I β-turn structure (Perczel and Hollosi, 1996) in SDS solution. In contrast, PMAP1 and PMAP2 revealed a partial α-helical structure in TFE solution, as compared to PMAP-23 (Fig. 2).

Discussion

PMAP-23 showed strong antibacterial activity against Gram-positive and Gram-negative strains (MIC: 3.1-12.5 μM). It displayed growth inhibitory activity against four different transformed cancer cells at 25-30 μM. In contrast, this peptide did not exhibit lytic activity against human erythrocytes at a concentration of 100 μM, suggesting it has certain degree of specificity toward the target cell membrane. Accordingly, PMAP-23 derived from pig myeloid cells is an important component of the leukocyte defense system.

Furthermore, the effect of two Pro residues at position 12 and 15 of PMAP-23 on antibacterial, antitumor and phospholipid vesicle-disrupting activities was investigated by $Pro^{12} \rightarrow$ Ala (PMAP1) and $Pro^{15} \rightarrow$ Ala (PMAP2) substitutions. These substitutions in PMAP-23 did not cause significant effect on antibacterial activity. This result may be due to the presence of the cell wall barrier outside of bacterial cell membrane, unlike eukaryotic cells. The rank order of antitumor and phospholipid vesicle-disrupting activities of the peptides was PMAP-23 > PMAP1 > PMAP2. These results suggest that two Pro residues in PMAP-23 are important for the phospholipid vesicle-disrupting activity as well as the growth inhibition of tumor cells. Also, Pro^{15} residue in PMAP-23 was more important in antitumor and vesicle-disrupting activities than Pro^{12} residue. PMAP-23 and its analogues displayed the typical type I β-turn conformation in SDS solution of the cell membrane mimicking environment. Ala substitution of position 12 or 15 in PMAP-23 induced a somewhat α-helical structure with a negative ellipicity at 222 nm in TFE solution. These results suggest that β-turn structure of PMAP-23 may has a positive effect on the antibiotic activity. Therefore, the tertiary structure of PMAP-23 and its analogues on the lipid membrane-mimicking condition should be determined using 2D-NMR analysis to elucidate the structure-antibiotic activity relationships.

In conclusion, PMAP-23 has a potent antitumor activity

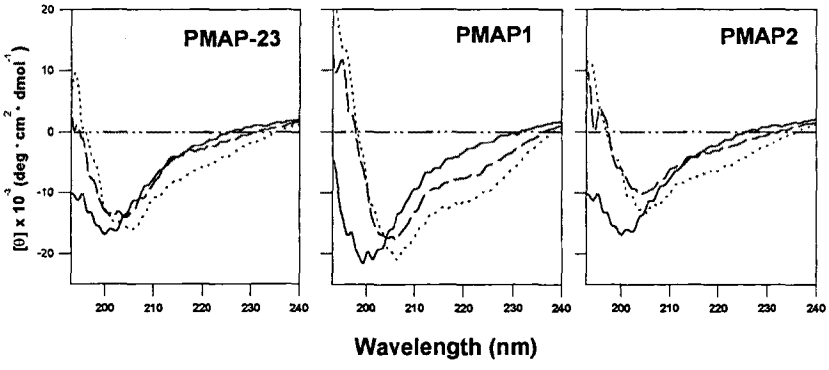

Fig. 2. CD spectra of PMAP-23 and its analogues in 10mM sodium phosphate buffer, pH 7.2 (—), 50 % (v/v) TFE (------) and 30 mM SDS (- - -) containing 10 mM sodium phosphate buffer, pH 7.2.

against various tumor cells as well as an antibacterial activity. In contrast, PMAP-23 did not induce hemolysis at 100 μM of the peptide. These results suggest that PMAP-23 is an antimicrobial peptide with the target cell specificity such as cecropins and magainins. PMAP-23 displays the type I β-turn structure on lipid membrane-mimicking environment such as SDS micelle. Two Pro residues of positions 12 and 15 in PMAP-23 play important roles in the β-turn structure on lipid membrane. The β-turn structure of PMAP-23 may be essential for antibiotic activity including phospholipid vesicle-disrupting activity.

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