Novel Synthetic Antibiotic Peptide derived from N-terminus of *Helicobacter pylori* Ribosomal Protein L1(RPL1)

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HP (2-20) (AKKVFKRLEKLFSKIQNDK) is the antimicrobial sequence derived from N-terminus of *Helicobacter pylori* Ribosomal Protein L1 (RPL1). To develop the novel antibiotic peptides useful as therapeutic antibiotic agents requires potent antibiotic activity against bacterial, fungi and cancer cells without cytotoxic effect. To this goal, several analogues with amino acid substitution were designed to increase or decrease only net hydrophobic region. In particular, substitution of Gln and Asp for hydrophobic amino acid, Trp at position 17 and 19 of HP (2-20) (Anal 3) caused a dramatic increase in antibiotic activity without hemolytic effect.

In contrast, the decrease of the hydrophobicity by substituting with Leu and Phe for Ser at position 12 and 19 of HP (2-20), respectively (Anal 4, Anal 5), did not have a significant effect on antibiotic activity. To compared of HP (2-20) and Anal 3 against fungal cell membranes was examined by treating prepared protoplasts of *C. albicans* and artificial liposomal vesicle (PC/PS; 3:1, w/w) disrupting activity test. The results showed that the Anal 3 more prevented the regeneration of fungal cell walls and induced enhanced the release of the fluorescent dye (Carboxyfluorescein) trapped in the artificial membrane vesicles than HP (2-20).

The potassium released test on *C. albicans* indicated the amounts of released potassium ion by the Anal 3 was more induced than parent peptide. These results indicated that the hydrophobic region of peptides is requisite for its effective antibiotic activity and may facilitate easily to penetrate the lipid bilayers of the cell membrane.

Additional CD spectra studies suggested that the a-helical structure of the peptides plays an important role in antibiotic effect but the a-helical property is less connected with the enhanced antibiotic activity.

Results and Discussion

Design and synthesis of the peptides

The amphipathic feature of a-helical antimicrobial peptides plays important role against target cells. A number of parameters, furthermore, in cluding net positively charge, a-helicity, overall hydrophobicity have been shown to modulate the antibiotic activity of the a-helical amphipathic antimicrobial peptides. Recent investigations using the amphipathic a-helical model peptides revealed that the peptide having hydrophilic amino acid residue and hydrophobic residues with ratios 9:9 possess potent anti microbial activity with no hemolytic effect. But hydrophobic residues with ratio 11:7 or 13:5 showed a potent hemolytic activity as well as antimicrobial activity. These results suggested that a moderate hydrophilic-hydrophobic balance of the a-helical antimicrobial peptides is a crucial factor in designing of the novel

antibiotic peptides having potent antibiotic activity with no cytotoxicity. HP (2-20) has hydrophobic and hydrophilic region as shown in a-helical wheel diagram.

Therefore, to elucidate the relationship of hydrophobic-hydrophilic region of antibiotic peptides, and to design novel peptides with more potent antibiotic activity than HP (2-20) without cytotoxicity, certain analogues were designed and synthesized. These analogues were designed by analysis of the a-helical wheel diagram of HP (2-20). The increase of the hydrophobicity by substitution with tryptophan which has hydrophobic property and the decrease of the hydrophobicity by substitution with serine. The synthetic peptides were purified by the reverse-phase HPLC and quantitated by amino acid analysis. The correct molecular weight of the synthetic peptides were confirmed by MALDI mass spectrometry.

HP (2-20) had been shown to inhibit the growth of Gram-positive and Gram-negative bacteria and fungi but no cytotoxicity against human erythrocytes. Also, Melittin, a honeybee venom toxin, has been reported to have a potent antimicrobial activity . While there had been extensive studies on the effects of this HP (2-20) on lipid vesicles and its effect on fungal cell membranes had ignored (unpublished data).

Peptides separate on RPHPLC due to their different hydrophobic interaction with C-18 of the stationary phase. Such hydrophobic interactions can be considered to be comparable to the interaction of amphipathic antimicrobial peptides with the lipid bilayer of plasma membranes. To investigate a possible correlation between over all peptide hydrophobicity and biological activities on microbial, cancer and human erythrocyte membrane, the hydrophobic characteristics of the peptides were investigated by using differential retention times on RPHPLC.

Antimicrobial and hemolytic activities

Antibacterial activities of the synthetic peptides against Gram-positive and Gram-negative bacterial strains determined as minimal inhibitory concentration (MIC) by the microdilution method. Each and all of Trp-substitution at position 17, 19 in HP (2-20) (Anal 1,2,3) resulted in improved antibacterial activity as compared with HP (2-20). In paticular, all of substitution of Gln and Asp for hydrophobic amino acid, Trp at position 17 and 19 of HP (2-20) (Anal 3) caused a dramatic increases in antibacterial activity. The time course of the synthetic peptides to kill mid-logarithmic phase of *E. coli* and *B. subtilis* were compared. Anal 3 and melittin, as positive control, displayed faster bactericidal rate against both *E. coli* and *B. subtilis* than other synthetic peptides. This fact suggests that the increased of hydrophobicity of synthetic peptides have an important role in the bactericidal rate than antibacterial activity. Antifungal activity of the peptides against the pathogenic fungi, was measured as MIC by MTT assay. The result showed that Anal 3 displayed about 10-fold greater antifungal activity than the parent HP (2-20) against all test microorganism. In order to visualize the fungicidal effect, the pathogenic fungus, *C. albicans* was treated with the peptide and spread on the YPD agar plate. As shown in Fig. 4, the peptide inhibited the growth of *C. albicans*. The order of antimicrobial activity of the synthetic peptides used in this study was Anal 3 > Anal 2 = Anal 1 > HP (2-20) > Anal 4 = Anal 5.

Previously, Magainin 2, Cecropin A or Cecropin B were toxic to cancer cells at concentrations lower than required to lyse normal fibroblast or human erythrocytes. Therefore, the anticancer activity of the peptides designed in this study against AML-2, SNU638, and SNU668 cancer cells was examined by MTT assay. As shown in Fig. 6, the HP (2-20) has almost no anticancer activity against three cancer cells. Meanwhile, Anal 3 has potent anticancer activity which is similar to melittin as positive control. In order to assess the cytotoxicity of the peptides against mammalian cell, the hemolysis percentage

was measured against human erythrocyte cells at various peptide concentrations, and the synthetic peptides showed no hemolytic activity, while melittin, as a positive control, exhibited a strong hemolytic activity. These results demonstrated that the HP (2-20) has a remarkable antibiotic activity but no hemolytic activity.

Structural Analysis of the Peptides by CD Measurements

In order to investigate the relation ship of structure and antibiotic activity of the peptides on lipids, the CD spectra of the peptides in phosphate buffer, and TFE solution or SDS micelles as lipid membrane-mimicking environment was measured. All peptides showed a random coil structure in an aqueous solution, while displayed the typical a-helical spectrum with two minimum peaks at 208 and 222nm in 50% TFE or 30mM SDS solution. HP (2-20) showed higher a-helical contents than the other analogue peptides in 50% TFE solution. In contrast, although Anal 3 has more potent antibiotic activity in bacterial, fungal, and cancer cells than HP (2-20), it displayed lower a-helicity than HP (2-20). These results suggested that the a-helical structure of the peptide may an important role in killing bacterial, fungal, and cancer cells, but the a-helical content is not correlated with the enhanced antibiotic activity.