Bacteria-Selective Synergism between the Antimicrobial Peptides α-Helical Magainin 2 and Cyclic β-Sheet Tachyplesin I: Toward Cocktail Therapy†

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ABSTRACT: Magainin 2 and tachyplesin I (T-SS) are membrane-permeabilizing antimicrobial peptides discovered from frog skin and horseshoe crab hemolymph, respectively. They are classified into different secondary structural classes, i.e., α-helix and cyclic β-sheet, respectively. We found that F5W-magainin 2 (MG2) and T-SS exhibited marked synergistic effects against Gram-negative and Gram-positive bacteria without enhancing hemolytic activity as a measure of toxicity. Dye release experiments using liposomes suggested that the selective synergism is mainly due to anionic phospholipid-specific synergism in membrane permeabilization. Furthermore, the cyclic structure of T-SS was found to be necessary for synergism because a linear analogue of T-SS did not show good synergism with MG2. These novel observations suggested the possibility of the development of cocktail therapeutic regimens using combinations of antimicrobial peptides.

Recently, the appearance of a growing number of bacteria that are resistant to conventional antibiotics has become a serious medical problem. To overcome this, development of antibiotics with novel mechanisms of action is an urgent issue (*1*). Endogenous antimicrobial peptides are promising candidates as novel antibiotics because they exhibit broad antimicrobial spectra and highly selective toxicities, and it is difficult for bacteria to develop resistance to these agents (*2*−*4*). Indeed, extensive studies have been carried out on the mechanisms of action (*5*−*8*), structure−activity relationships (*9*−*13*), and effective mass production of these antimicrobial peptides (*14*, *15*).

Combined use of antibiotics that act synergically could reduce the dose and side effects and prevent development of resistant bacteria (*16*, *17*). A number of examples of synergism between conventional antibiotics and antimicrobial peptides have been reported (*18*, *19*). In contrast, there have been fewer reports of synergism between antimicrobial peptides. Such synergism has usually been observed between peptides of the same secondary structural class and origin, suggesting some biological significance. Representative examples are synergism between magainin 2 and PGLa (*20*) and between dermaseptin congeners (*21*). However, these peptide combinations may not be appropriate for combination therapy because their toxicities are also enhanced by coadministration.

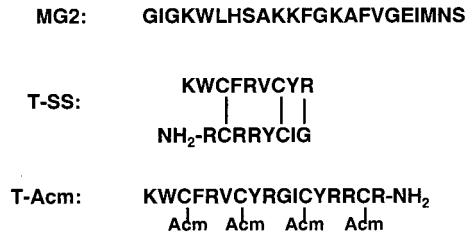

FIGURE 1: Amino acid sequences of the peptides used in this study.

Here, we report a novel and unique combination of antimicrobial peptides of different structure classes. α-Helical F5W-magainin 2 (MG2)[1] from frog skin (*6*, *7*, *22*) and cyclic β-sheet tachyplesin I (T-SS) from horseshoe crab hemolymph (Figure 1) (*7*, *23*) were found to show marked synergism against Gram-negative and Gram-positive bacteria without enhancing hemolytic activity as a measure of toxicity. Furthermore, the cyclic structure of T-SS was found to be necessary for synergism, because a linear analogue of T-SS (T-Acm, Figure 1) (*24*−*26*) did not exhibit good synergism with MG2.

MATERIALS AND METHODS

Materials. The peptides were synthesized by Fmoc-based solid-phase synthesis and authenticated as described previously (*27*, *28*). EYPG, EYPC, CCCP, and polymixin B

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NPN, *N*-phenyl-1-naphthylamine; EYPC, egg yolk L-α-phosphatidylcholine; EYPG, L-α-phosphatidyl-DL-glycerol enzymatically converted from EYPC; FIC, fractional inhibitory concentration; Fmoc, fluoren-9-ylmethoxycarbonyl; LUVs, large unilamellar vesicles; MG2, F5W-magainin 2; MIC, minimal inhibitory concentration; MLVs, multilamellar vesicles; PC, phosphatidylcholine; PG, phosphatidylglycerol; T-Acm, acyclic tachyplesin I analogue with the four SH groups of tachyplesin I protected by acetamidomethyl groups; TSB, trypticase soy broth; T-SS, tachyplesin I; cfu, colony-forming unit.

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sulfate were obtained from Sigma (St. Louis, MO). NPN was purchased from Kanto Chemical (Tokyo, Japan). Calcein and spectrograde organic solvents were supplied by Dojindo (Kumamoto, Japan). All other chemicals were special grade and were obtained from Wako. The buffers were prepared with double-distilled water. Phosphate-buffered saline (pH 7.4) for hemolysis measurements was purchased from Sigma.

Antimicrobial Activity. The MICs of the peptides were determined as described elsewhere (*29*). *Escherichia coli* (ATCC 25922) and *Staphylococcus epidermidis* (ATCC 12228) were cultured in 3% (w/v) TSB at 37 °C overnight. To obtain midlogarithmic phase microorganisms, 1 mL aliquots of the cultures were then transferred to 100 mL of fresh TSB broth and incubated for 2−3 h. The cells were washed with buffer [10 mM sodium phosphate and 100 mM NaCl (pH 7.4)] and resuspended in the same buffer. The cell concentrations were estimated by measuring the absorbance at 600 nm (ABS_{600}). The relationships between cell concentration and ABS_{600} were predetermined as cfu/mL = $(ABS_{600})(3.4 \times 10^8)$ and cfu/mL = $(ABS_{600})(1.6 \times 10^8)$ for *E. coli* and *S. epidermidis*, respectively. The suspensions were diluted to 4×10^5 cfu/mL. The inoculum (90 μL) was added to each well of 96-well plates. The peptide samples (10 μL) were added to each well and the plates incubated at 37 °C for 3 h. TSB [6% (w/v), 100 μL] was added, and the plates were incubated at 37 °C for 20 h. Cell growth was assessed by measuring the optical density of the cultures at 620 nm on a model 550 microplate reader (Bio-Rad, Hercules, CA). MIC was defined as the lowest concentration of peptide that inhibited growth.

Calcein Leakage. LUVs were prepared and characterized as described elsewhere (*30*). Briefly, a lipid film, after drying under vacuum overnight, was hydrated with a 70 mM calcein solution (pH adjusted to 7.4 with NaOH) or HEPES/NaOH buffer [10 mM HEPES, 150 mM NaCl, and 1 mM EDTA (pH 7.4)] and vortex-mixed to produce MLVs. The suspensions were subjected to five cycles of freezing and thawing and then successively extruded through polycarbonate filters (100 nm pore size filter, 21 times). The lipid concentration was determined in triplicate by phosphorus analysis (*31*). Vesicles containing calcein were separated from free calcein on a Bio-gel A-1.5m column. If necessary, calcein-free LUVs were mixed with dye-loaded liposomes to adjust the lipid concentration to the desired value. The release of calcein from the LUVs was fluorometrically monitored on a Shimadzu RF-5000 spectrofluorometer at an excitation wavelength of 490 nm and at an emission wavelength of 520 nm at 30 °C. The maximum fluorescence intensity corresponding to 100% leakage was determined by addition of 10% (w/v) Triton X-100 (20 μL) to 2 mL of the sample. The apparent percent leakage value was calculated according to the relationship % apparent leakage = $100(F - F_0)/(F_t - F_0)$, where F and F_t are the fluorescence intensity before and after addition of the detergent, respectively, and F_0 represents the fluorescence of intact vesicles.

NPN Uptake Assay. The outer membrane permeabilization activity of peptides was investigated with an NPN uptake assay (*18*, *32*). Briefly, 1 mL of an overnight culture of *E. coli* (ATCC 25922) was added to 50 mL of fresh 3% (w/v) TSB medium and grown to an ABS_{600} of 0.4−0.6. The cells were washed with buffer [5 mM HEPES/NaOH, 5 mM glucose, 100 mM NaCl, and 5 μM CCCP (pH 7.4)] and resuspended in the same buffer. The cell concentration was determined as described above. For fluorescence measurements, 2 mL of cell suspension (ABS_{600} = 0.5) was prepared in a cuvette and 20 μL of a 1 mM NPN solution in acetone was added followed by 20 μL of an aqueous peptide solution. The fluorescence of NPN was monitored on a Shimadzu RF-5000 spectrofluorometer at an excitation wavelength of 350 nm and at an emission wavelength of 420 nm at 30 °C. The maximal value of NPN uptake was determined after addition of polymixin B sulfate (0.64 mg/mL, 10 μL).

Hemolytic Activity. Human erythrocytes (blood type O) from a healthy 26-year-old female were freshly prepared prior to the experiments. The blood was centrifuged (800*g* for 10 min) and washed three times with PBS (pH 7.4) to remove plasma and the buffy coat. Erythrocyte specimens were kept on ice throughout the experiments. Various concentrations of peptides were incubated with the erythrocyte suspension [final erythrocyte concentration, 1% (v/v)] for 1 h at 37 °C. The percent hemolysis was determined from the optical density at 540 nm of the supernatant after centrifugation (800*g* for 10 min), as described elsewhere (*33*). Hypotonically lysed erythrocytes were used as the standard for 100% hemolysis.

RESULTS AND DISCUSSION

Antimicrobial Activity. Synergism in antimicrobial activity was investigated against the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *S. epidermidis*. Table 1 shows the MIC values of each peptide alone or in combination with other peptides. The synergistic activity was expressed as the fractional inhibitory concentration (FIC) index. Although the interpretation of FIC index values differs slightly among researchers, there is consensus that an FIC index of ≤0.5 indicates good synergy (*34*, *35*). The FIC value of a peptide mixture was evaluated under two conditions. The MIC value of one peptide was determined in the presence of approximately $^1/_{10}$ of the MIC of the other peptide, and vice versa. The combination of MG2 and T-SS exhibited strong synergism against both Gram-positive and Gram-negative bacteria (FIC ∼ 0.3). We also used the linear derivative of tachyplesin I, T-Acm, to investigate the role of the cyclic structure of T-SS connected by two intramolecular disulfide bridges. FIC values larger than 0.5 indicated no good synergism in the combination of MG2 and T-Acm, suggesting the importance of the cyclic structure of T-SS for the synergistic effects.

Membrane Permeabilization Activity against Acidic Phospholipid-Containing Membranes. MG2 and T-SS are representative membrane-acting antimicrobial peptides, which are thought to kill bacteria by permeabilizing negatively charged cell membranes (*22*, *33*, *36*, *37*). Phosphatidylglycerol-containing lipid bilayers are a good model for bacterial membranes and have often been used to investigate the mechanisms of action of this class of peptides (*5*−*7*, *9*, *11*, *25*, *26*, *28*−*30*, *33*, *37*−*40*). Synergism in membrane permeabilization activity was investigated by the efflux of a fluorescent dye, calcein, entrapped within LUVs composed of EYPG and EYPC (1/1) under various conditions. Figure 2A shows typical data indicating that low concentrations of MG2 (trace 1) and T-SS (trace 2) alone did not induce significant dye release, whereas their mixture (trace 3)

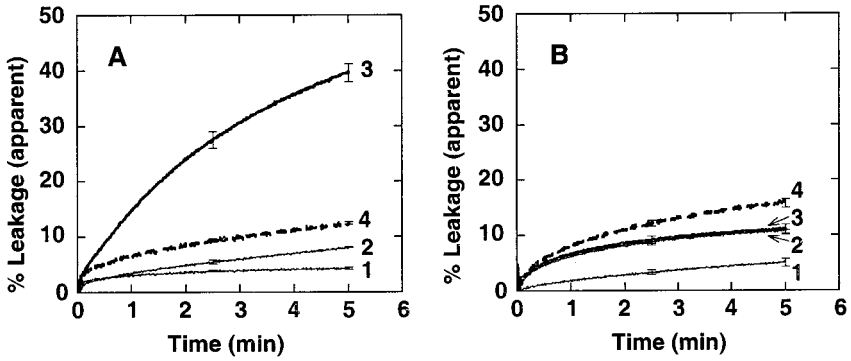

FIGURE 2: Membrane permeabilization activity against negatively charged LUVs. The peptides were added to calcein-loaded LUVs composed of EYPG and EYPC (1/1). The dye release was fluorometrically detected as a function of time at 30 °C. (A) Trace 1, 0.575 μM MG2; trace 2, 0.275 μM T-SS; trace 3, a mixture of 0.575 μM MG2 and 0.275 μM T-SS; and trace 4, the arithmetic sum of traces 1 and 2; [lipid] = 242 μM. (B) Trace 1, 0.13 μM MG2; trace 2, 2.5 μM T-Acm; trace 3, a mixture of 0.13 μM MG2 and 2.5 μM T-Acm; and trace 4, the arithmetic sum of traces 1 and 2; [lipid] = 32 μM.

Table 1: Antimicrobial Activities of Peptides Alone or in Combination with Other Peptides

	MIC (μM)[a]		
	alone[c]	in combination	
bacteria	MG2	MG2 + T-SS	FIC index[b]
E. coli	20	1.25 + 0.1	0.26
S. epidermidis	40	1.25 + 0.1	0.23
	MIC (μM)[a]		
	alone[c]	in combination	
bacteria	T-SS	T-SS + MG2	FIC index[b]
E. coli	0.5	0.125 + 2	0.35
S. epidermidis	0.5	0.125 + 4	0.35
	MIC (μM)[a]		
	alone[c]	in combination	
bacteria	MG2	MG2 + T-Acm	FIC index[b]
E. coli	20	10 + 0.2	0.6
S. epidermidis	40	20 + 0.1	0.55
	MIC (μM)[a]		
	alone[c]	in combination	
bacteria	T-Acm	T-Acm + MG2	FIC index[b]
E. coli	2	1 + 2	0.6
S. epidermidis	2	1 + 4	0.6

[a] Minimal inhibitory concentration (MIC) against *E. coli* ATCC 25922 and *S. epidermidis* ATCC 12228. [b] The fractional inhibitory concentration (FIC) index was calculated as follows. FIC index = $[A]/MIC_A + [B]/MIC_B$, where MIC_A and MIC_B are the MICs of peptides A and B alone, respectively, and [A] and [B] are the MICs of peptides A and B in combination, respectively. [c] MIC values were determined by serial 2-fold dilutions of peptides. Therefore, MIC values within 2-fold were within experimental errors. We predetermined the MIC value of each peptide alone. Combination experiments were then carried out, and in parallel, the MIC of each peptide alone was redetermined as a control. The redeterimed MIC values of each peptide alone are shown.

markedly enhanced membrane permeabilization compared with the arithmetic sum (trace 4) of traces 1 and 2, suggesting strong synergism in membrane permeabilization activity. On the other hand, the activity of the MG2/T-Acm mixture was decreased compared with the arithmetic sum of those of individual peptides alone (Figure 2B). The latter observation is what is expected if the two peptides interact with the membrane independently, because charge neutralization of the membrane surface due to binding of one peptide prevents binding of the other to some extent. Thus, the results of membrane permeabilization activity against the negatively charged LUVs were fully consistent with the observed antimicrobial activity (Table 1).

Perturbation of the Outer Membrane. In the case of Gram-negative bacteria, outer membranes play a role as a drug barrier (*41*). Polycationic peptides were proposed to cross outer membranes by the "self-promoted uptake" mechanism (*41*). Therefore, the synergism between MG2 and T-SS in the outer membrane perturbing activity was investigated with an NPN uptake assay (*18*, *32*). NPN is normally excluded from outer membranes but is partitioned into perturbed outer membranes exhibiting increased fluorescence. Figure 3 shows that the MG2/T-SS (Figure 3A, trace 3) as well as MG2/T-Acm (Figure 3B, trace 3) mixtures exhibited NPN uptake close to the arithmetic sums (traces 4) of the curves of individual peptides alone (traces 1 and 2), although the initial rates were slightly enhanced. These results suggested that T-SS and T-Acm exhibit no synergism in outer membrane-disturbing activity and that the mechanism of synergism of the T-SS/MG2 pair is related to the effects of the peptides on the cell membrane.

Hemolytic Activity. Even if a combination of peptides exhibits strong synergism, it cannot be applied to clinical use if toxicity is also enhanced. Therefore, we estimated the hemolytic activity as a measure of toxicity at peptide concentrations 100-fold higher than MICs against *S. epidermidis*. Although T-SS alone inhibited the growth of bacteria at a much lower concentration (0.5 μM) than MG2 (40 μM), T-SS also exhibited hemolytic activity as strong as that of MG2 at 100 × MIC (Figure 4A, cross-hatched and striped bars); i.e., the therapeutic indices were similar. In the combined use of MG2 and T-SS, although antimicrobial activity was increased (Table 1), hemolytic activity (Figure 4A, black bar) was much lower than those of individual peptides alone at 100 × MICs. It should be noted that the hemolytic activity of the mixture was approximately half of the arithmetic sum of those of the component peptides (Figure 4A, hatched and white bars).

For comparison, hemolytic activities of MG2 and PGLa were also investigated (Figure 4B). Magainin 2 and PGLa have been reported to exhibit strong synergism in various systems, including bacteria, tumors, and lipid vesicles, by the formation of a 1/1 molecular complex in membranes (*20*,

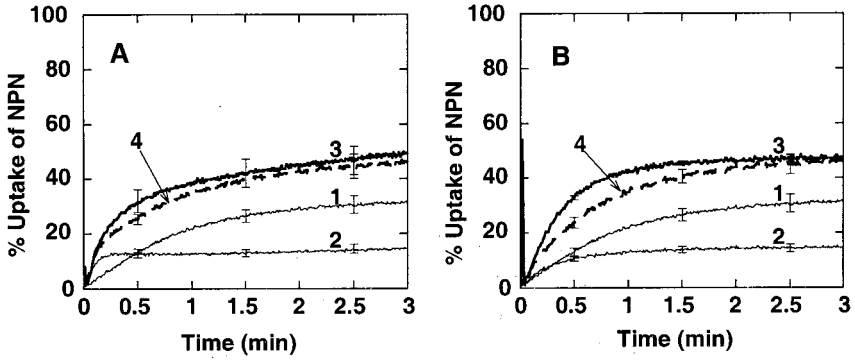

FIGURE 3: Outer membrane perturbing activity. The fluorescent dye NPN was added to *E. coli* cells, followed by addition of peptide. NPN uptake into outer membranes due to peptide-induced membrane perturbation was monitored as the increase in fluorescence at 30 °C (*18*, *32*). Time courses of NPN uptake are shown as traces. (A) Trace 1, 0.123 μM MG2; trace 2, 0.123 μM T-SS; trace 3, a mixture of 0.123 μM MG2 and 0.123 μM T-SS; and trace 4, the arithmetic sum of traces 1 and 2. (B) Trace 1, 0.123 μM MG2; trace 2, 0.123 μM T-Acm; trace 3, a mixture of 0.123 μM MG2 and 0.123 μM T-Acm; and trace 4, the arithmetic sum of traces 1 and 2.

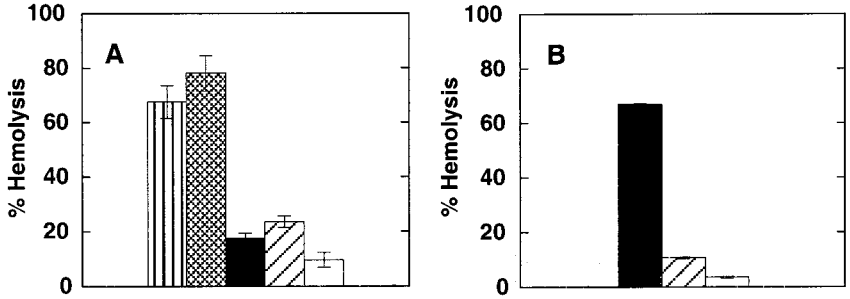

FIGURE 4: Hemolytic activities as a measure of toxicity. Peptides were incubated with 1% (v/v, final concentration) erythrocyte suspension for 1 h at 37 °C. Percent hemolysis was determined by measuring the absorbance of the supernatant after centrifugation. (A) Percent hemolysis values at 100 × MICs are shown as bars: striped, 4000 μM MG2; cross-hatched, 50 μM T-SS; and black, a mixture of 12.5 μM T-SS and 400 μM MG2. The values of individual peptides alone in the mixture are also shown by bars: hatched, 12.5 μM T-SS; and white, 400 μM MG2. (B) The percent hemolysis value of 125 μM (=100 × MIC) for a 1/1 mixture of PGLa and MG2 is shown by the black bar. The values of individual peptides alone in the mixture are also shown by bars: hatched, 62.5 μM PGLa; and white, 62.5 μM MG2.

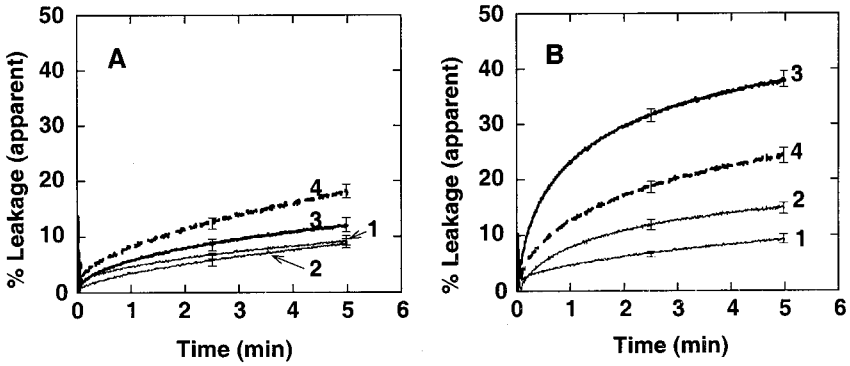

FIGURE 5: Membrane permeabilization activity against EYPC LUVs. The release of calcein was fluorometrically detected at 30 °C; [lipid] = 4.5 μM. (A) Trace 1, 1 μM MG2; trace 2, 1 μM T-SS; trace 3, a mixture of 1 μM MG2 and 1 μM T-SS; and trace 4, the arithmetic sum of traces 1 and 2. (B) Trace 1, 1 μM MG2; trace 2, 1 μM PGLa; trace 3, a mixture of 1 μM MG2 and 1 μM PGLa; and trace 4, the arithmetic sum of traces 1 and 2.

38). The MICs of MG2 alone, PGLa alone, and a 1/1 mixture were 20, 5, and 2.5 μM (FIC = 0.31) against *E. coli*, respectively, and 20−40, 2.5, and 1.25 μM (FIC = 0.28) against *S. epidermidis*, respectively. The FIC indices were similar to those of the MG2/T-SS pair (Table 1). However, for the MG2/PGLa pair, hemolytic activity was also as high as that of MG2 alone at 100 × MICs (Figure 4B, black bar, and Figure 4A, striped bar); i.e., the therapeutic index was not improved by the combination of MG2 and PGLa. Furthermore, the hemolytic activity of the MG2/PGLa mixture was much stronger than the arithmetic sum of those of the component peptides (Figure 4B, hatched and white bars).

Membrane Permeabilization Activity against Zwitterionic Phospholipid Membranes. The question of why the combination of MG2 and T-SS exhibited synergy only in antimicrobial activity is interesting. One of the major differences between bacterial plasma membranes and erythrocyte membranes is that the outer leaflets of erythrocyte membranes are almost exclusively composed of zwitterionic phospholipids (*42*), whereas bacterial membranes contain abundant acidic phospholipids (*43*). Therefore, membrane permeabilization activity was also investigated by use of EYPC LUVs (Figure 5). Both MG2 and T-SS permeabilized EYPC membranes at much higher peptide-to-lipid ratios composed

with negatively charged membranes (Figure 2A), in keeping with the bacteria-selective properties of these peptides. Consistent with the hemolytic activity (Figure 4), the dye leakage activity of the MG2/T-SS mixture (Figure 5A, trace 3) was lower than the arithmetic sum (trace 4) of those of the components alone (traces 1 and 2). In contrast, the MG2/PGLa combination also exhibited marked synergism in the permeabilization of EYPC bilayers (Figure 5B).

The observations that MG2 and T-SS exhibited synergism in membrane permeabilization activity only against negatively charged phospholipid membranes suggested that the mechanisms of permeabilization of PC (erythrocyte) membranes and bacterial outer membranes may differ from those of PG-containing (bacterial cell) membranes. The membrane permeabilization mechanisms of MG2 and T-SS against negatively charged phospholipid membranes have been extensively investigated (*6*, *7*, *25*, *26*, *28*, *30*, *37*, *39*, *40*, *44*). Magainin is considered to form a transient peptide−lipid supramolecular complex pore of definite size (2−3 nm in diameter) in PG-based membranes. This mechanism is unlikely to be present in outer membranes because of the presence of bulky polysaccharide chains of lipopolysaccharides. Furthermore, magainin peptides have been suggested to permeabilize PC bilayers by perturbing bilayer organization rather than pore formation (*45*).

Selective Synergy. In this study, we discovered that a novel combination of antimicrobial peptides, T-SS and MG2, with different structures and origins exhibited strong synergy in antimicrobial activity (Table 1) but not in hemolytic activity (Figure 4), although the mechanism of this selective synergy is not yet clear. The synergy, for which the cyclic structure of T-SS is essential (Table 1 and Figure 2B), appears to be closely related to selectively synergistic permeabilization of acidic phospholipid-containing membranes (Figure 2). Recently, Hancock's group reported synergistic interactions between antimicrobial peptides of different structural classes (*46*), although data on toxicity were not reported. Strong synergism was also found between α-helical LL-37 and cyclic β-sheet protegrin 1. Our observations were in marked contrast to the nonselective synergism between MG2 and PGLa (Figure 4 and refs *20* and *38*). Discovery of peptide combinations with selective synergism will open up the possibility of cocktail therapy and may answer the question of why a single animal produces many antimicrobial peptides with different structures.

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