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Tuning the Activity of Anoplin by Dendrimerization of Lysine and Lipidation of the N-Terminal

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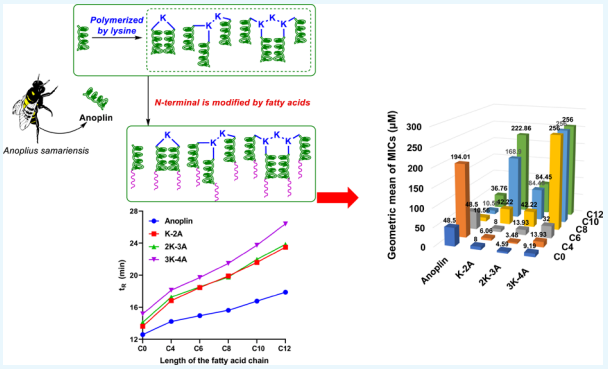
ABSTRACT: Dendrimeric antimicrobial peptides or lipopeptides have strong transmembrane ability and antibacterial activity. To obtain some ideal antimicrobial peptides, anoplin, a natural antimicrobial peptide with weak antimicrobial activity, was modified by C-terminal dendrimerization using lysine and N-terminal lipidation using fatty acids. 2K-3A-C4, a trimer of anoplin, was dendrimerized by two lysines at the C-terminal and was lipidated by *n*-butyric acid at the N-terminal, and thus exhibited the best antibacterial activity. However, the trimer had high hemolytic activity. Finally, A-C8, a simple structural lipopeptide, which is not a dendrimer, was obtained following the lipidation of anoplin using octanoic acid; it exhibited the highest therapeutic index, which makes it a probable antibiotic and thus was screened out.



INTRODUCTION

Common bacterial infections are easy to cure; however, multidrug-resistant bacteria complicate the treatment of bacterial infections, which are caused by the abuse of antibiotics.[1] To date, the commonly used clinical antibiotics are still those produced from molecules developed in the 1960s, and the original targets of these antibiotics have undergone significant changes in bacterial cells, thereby causing a decline in their efficacy. The emergence of antimicrobial peptides (AMPs) has brought hope to solve the problem of bacterial resistance.[2] AMPs, which are part of the immune defense system of an organism, are a class of small molecular peptides with broad-spectrum antimicrobial activity. They have a significant killing effect on multidrug-resistant bacteria and can interact with the immune system; also, they can act directly on the bacterial membrane, thereby causing physical damage, and have multiple sterilization mechanisms and multiple targets to quickly kill bacterial organisms. Because the structure and surface charge-related performance of bacteria remain unchanged, drug resistance is difficult to develop in AMPs.[3−6]

Anoplin, a natural AMP, extracted from the venom of the wasp *Anoplius samariensis*, is a short peptide composed of 10 amino acids. It is also the shortest amphipathic α-helical AMP found so far.[7] It is gratifying that anoplin has a high α-helix content in an environment simulated by bacterial membranes.[8] anoplin exhibits potential antibacterial activity and low hemolytic activity on mammalian erythrocytes. However, it has poor stability and is easily degraded by proteases, which affect its bioavailability.[9] In our previous study, to improve the stability and activity of anoplin, the strategy of modification used is to add propargylglycine (Pra) and azide lysine to the N-terminus of two anoplins and then conduct "click chemistry" for linking and heteropolymerization using a triazole. This strategy significantly improved the antibacterial activity and stability of the enzymatic hydrolysis of anoplin.[10] Later, we modified anoplin with different lengths of fatty acid (C4−C12) and used the triazole-linking strategy to heteropolymerize. The stability and activity of these analogues were significantly improved.[11,12]

RESULTS AND DISCUSSION

The dendrimerized anoplin was a hyperbranched dendrimer molecule. The hyperbranched peptide dendrimers have multivalency, which is one of the unique features of the dendrimers, this is prevalent in biology and refers to multiple simultaneous binding interactions between the receptor and the ligand. Multivalent ligands on the dendritic scaffold can bind multiple receptors and hence increase the binding.[13] In this study, anoplin was heterodendrimerized using lysine at the C-terminus to form a dimer, a trimer, and a tetramer of anoplin, and they were lipidated using *n*-butyric acid, hexanoic acid, octanoic acid, decanoic acid, and lauric acid at the N-terminus (Figure 1). Then, the effects of the degree of dendrimerization and fatty chain length of these anoplin variants on their antibacterial properties were explored.

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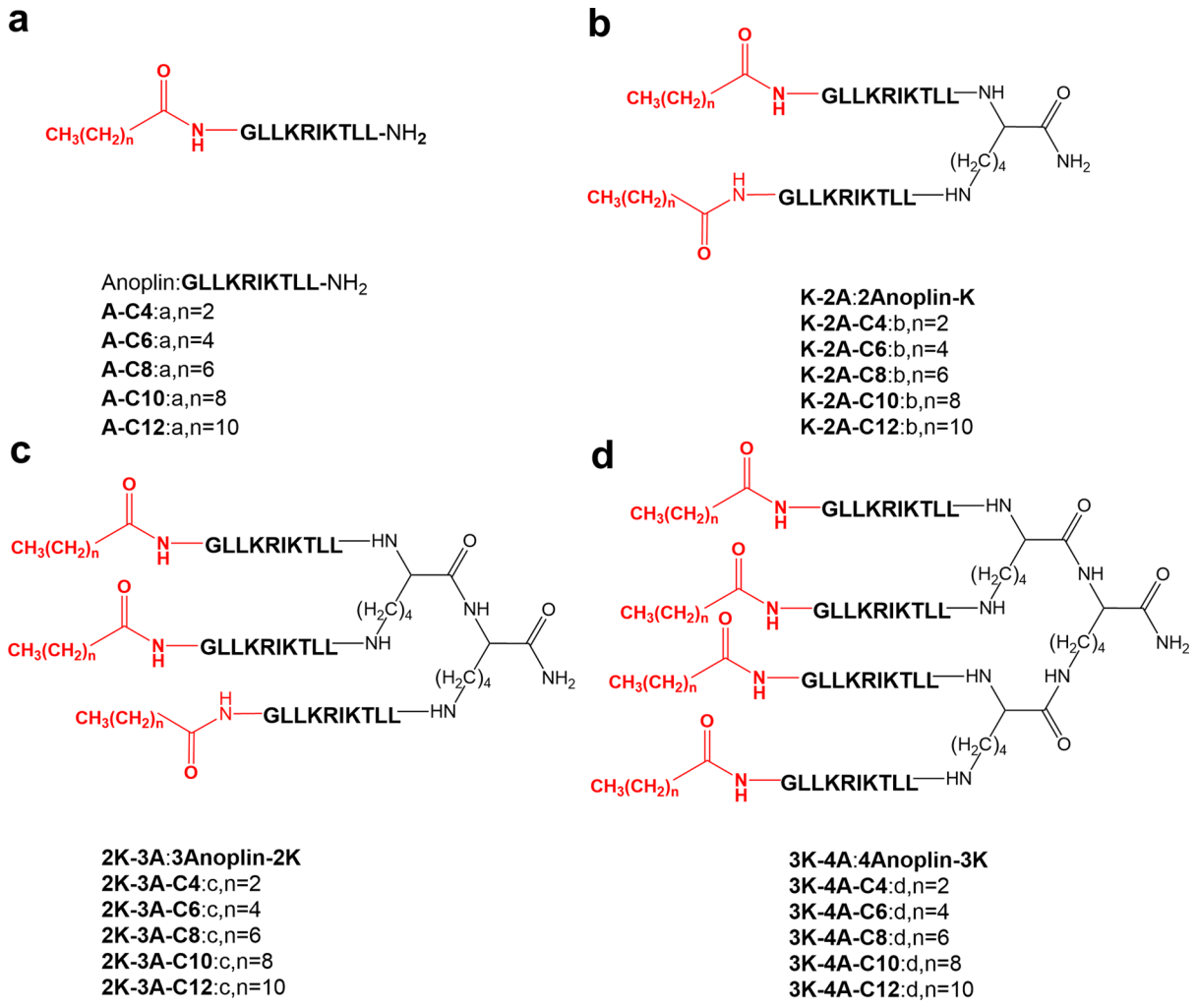

Figure 1. Anoplin analogues dendrimerized with lysine and lipidated with a linear chain fatty acid at the N-terminal. (a) Single anoplin, (b) dimer dendrimerized by two anoplin and one lysine, (c) trimer dendrimerized by three anoplin and two lysines, and (d) tetramer dendrimerized by four anoplin and three lysines. They were lipidated by *n*-butyric acid, hexanoic acid, octanoic acid, decanoic acid, and lauric acid at the N-terminal.

The synthesis of these peptides was performed using established Fmoc-based solid-phase peptide synthesis procedures (Figure 2). The introduction of a highly acid-labile 4-methyltrityl (Mtt) protected the lysine residue and allowed for selective dendrimerization of the side-chain amino-group. Then, short-chain fatty acid anhydrides or long-chain fatty acids were coupled using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIPEA) in *N*′,*N*-dimethylformamide (DMF). After cleavage of the lipidated peptides from the resin, all batches of products were identified using electrospray ionization-mass spectrometry (ESI-MS). They were purified by preparative high-performance liquid chromatography (HPLC) to more than 95% purity, lyophilized, and tested for antibacterial activity (Tables 1 and S1). Their hydrophobicity is expressed as the retention time (t_R) on HPLC. Their t_R increased with the degree of dendrimerization or fatty acid chain length. To determine the secondary structure of the new peptides, their circular dichroism (CD) in water and in a simulated environment of bacterial membranes (50% trifluoroethanol, TFE) has been determined (in Figure 3), and their α-helical content was calculated (results shown in Table 1). Anoplin, A-C4, A-C6, A-C8, A-C10, and A-C12 did not show a α-helical structure in a water environment. In the dimers, only K-2A-C12 showed an α-helical structure (α-helical content is 60.70%) in a water environment. In the trimers, 2K-3A-C10 and 2K-3A-C12 showed an α-helical structure (α-helical contents are 67.04 and 82.89%, respectively) in a water environment. In the tetramers, 3K-4A-C8, 3K-4A-C10, and 3K-4A-C12 showed an α-helical structure (α-helical contents are 47.72, 61.58, and 70.83%, respectively) in a water environment. These suggest that the α-helical content of the anoplin analogue in water can be increased by dendrimerizing or modifying with fatty acids. Their antibacterial activities of the prepared peptides were tested against a representative panel of pathogenic bacteria, including one strain of *Staphylococcus aureus* (Gram positive) and *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Gram negative); the nonpathogenic Gram-positive *Bacillus subtilis* was also included. As mentioned before, we were particularly interested in peptides that showed activity against *P. aeruginosa* and *S. aureus*. For comparison, along with the 15 lipopeptides, the unmodified lysine-dendrimerized-peptide and anoplin were included in the activity screening.

Minimal inhibitory concentrations (MICs) were determined according to the CSLI guidelines.[14] Briefly, serial dilutions of peptides were prepared in Mueller-Hinton broth (MHB). The cultures were inoculated with 1×10^6 CFU/mL and incubated at 37 °C for 18 h. The lowest concentration that inhibited visible

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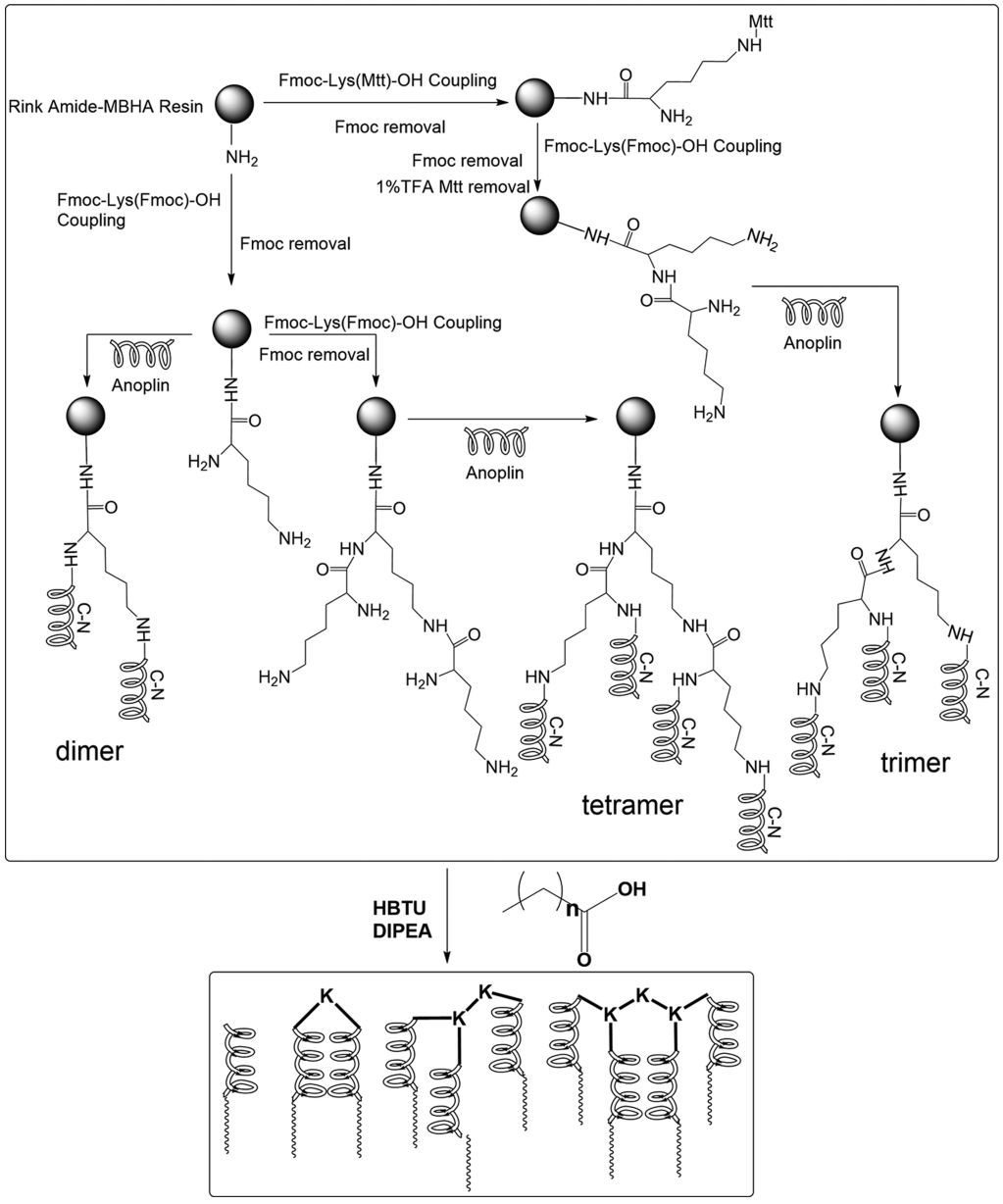

Figure 2. Synthesis of multivalent anoplin analogues. In the dendrimerized peptides, their anoplin was individually synthesized by Fmoc-amino acids instead of adding synthesized anoplin.

growth is reported as the MIC. Tests were independently performed three to five times.

The general trend is that dendrimerized lysine and lipidated fatty acid will increase the activity of anoplin (Table 1 and Figure 4). In fact, for single anoplin analogues, which have good antibacterial activity after attachment to the fatty acid of C8−C12. The highest activity against various pathogens was found for compounds attached with octanoic acid or decanoic acid. For example, the introduction of the C-terminal acylated by C8 or C10-lipids increased the activity 40-fold against *S. aureus*. The peptides lipidated with C4−C6 and C12 lowered antibacterial activity due to low hydrophobicity or poor solubility in the medium used for the MIC test. Interestingly, in the same chain length fatty acid-modified dendrimers, as the degree of dendrimerization increased, their activity increased at first and then decreased, also, the antibacterial activity of the trimer was higher than those of the dimer and tetramer. The fatty acid modification also showed a similar trend. As the length of the fatty acid chain increased, the activity of the analogues also increased at first and then decreased. Among these analogues, 2K-3A-C4, the trimeric anoplin dendrimerized by two lysines and three anoplin after being modified with *n*-butyric acid, exhibited the best activity (GM = 3.48 μM, GM is the geometrical mean of the MIC of peptides). The possible mechanism is that as the degree of dendrimerization increases, the hydrophobicity, cationic nature, and multivalency of the dendrimer will increase, and the trimer will reach its optimal state. However, the tetramer formed a conformation, which is not conducive to antibacterial activity due to its too strong hydrophobicity and large molecular volume.[15,16] From the CD data, K-2A-C12, 2K-3A-C10, 2K-3A-C12, 3K-4A-C8, 3K-4A-C10, and 3K-4A-C12 have a higher α-helical content than other analogues in water, but they have lower antimicrobial activity (GM > 42 μM). Therefore, the high α-helical content of anoplin analogues in water may have a negative effect on the antimicrobial activity but no obviously regular effect on

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Table 1. Design of Peptides and Their Antibacterial Activity and Key Physicochemical Parameters

	MIC[a] of Gram-negative bacteria (μM)		MIC of Gram-positive bacteria (μM)									
peptide	*S. aureus* ATCC 25923	*B. subtilis* ATCC 23857	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*K. pneumoniae* ATCC 700603	GM[b] (μM)	MHC[c] (μM)	IC_{50} for GMC[d] (μM)	TI[e]	t_R[f] (min)	α-helix in water (%)	α-helix in 50% TFE (%)
Anoplin	256	8	128	8	128	48.50	>256	>256	10.56	12.599	14.03	82.64
A-C4	256	256	256	64	256	194.01	>256	>256	2.64	14.234	16.68	86.47
A-C6	64	16	128	16	128	48.50	>256	>256	10.56	14.953	21.37	86.74
A-C8	8	8	16	8	16	10.56	210.63	>256	19.95	15.629	21.07	91.01
A-C10	8	8	16	8	16	10.56	16.36	184.55	1.55	16.764	18.22	83.24
A-C12	16	16	64	32	128	36.76	11.38	>256	0.31	17.885	26.72	80.09
K-2A	16	4	8	8	8	8.00	51.2	61.89	6.40	13.627	13.60	80.56
K-2A-C4	8	4	4	8	8	6.06	27.61	58.22	4.55	16.825	22.73	77.00
K-2A-C6	8	8	4	16	8	8.00	6.08	42.73	0.76	18.460	22.05	77.51
K-2A-C8	64	32	16	128	32	42.22	5.67	29.43	0.13	19.901	25.16	76.25
K-2A-C10	256	256	64	256	128	168.90	26.56	57.69	0.16	21.596	29.34	76.88
K-2A-C12	256	256	128	256	256	222.86	15.16	102.13	0.07	23.492	60.70	84.15
2K-3A	4	4	8	4	4	4.59	2.53	3.73	0.55	14.133	14.73	98.35
2K-3A-C4	4	4	4	2	4	3.48	3.18	2.05	0.91	17.270	21.70	83.97
2K-3A-C6	16	8	16	8	32	13.93	0.71	1.37	0.05	18.539	25.60	77.83
2K-3A-C8	32	32	32	32	128	42.22	59.73	164.3	1.41	19.773	32.59	75.15
2K-3A-C10	128	256	128	32	32	84.45	146.63	>256	1.74	21.973	67.04	84.76
2K-3A-C12	64	256	64	64	64	84.45	103.52	>256	1.23	23.843	82.89	84.01
3K-4A	8	8	8	8	16	9.19	1.31	1.69	0.14	15.178	16.35	71.32
3K-4A-C4	16	16	8	8	32	13.93	1.06	1.28	0.08	18.133	32.70	81.52
3K-4A-C6	32	32	32	32	32	32.00	10.91	2.36	0.34	19.716	28.39	89.64
3K-4A-C8	256	256	256	256	256	256.00	103.40	31.89	0.40	21.480	47.72	96.73
3K-4A-C10	256	256	256	256	256	256.00	12.51	2.73	0.05	23.739	61.58	94.71
3K-4A-C12	256	256	256	256	256	256.00	210.92	116.32	0.82	26.406	70.83	88.10

[a]Minimum inhibitory concentration (MIC, μM) was determined as the lowest concentration of a peptide that inhibited 95% of the bacterial growth. Data are representative of three independent experiments. [b]The geometric mean (GM) of the peptide MICs against bacteria and fungi was calculated. When no detectable antimicrobial activity was observed at 256 μM, a value of 512 μM was used to calculate the TI. [c]MHC is the minimum hemolytic concentration that caused 10% hemolysis of human erythrocytes. Data are representative of three independent experiments. When no detectable hemolytic activity was observed at 256 μM, a value of 512 μM was used to calculate the TI. [d]IC_{50} for GMC is the concentration of peptide corresponding to when the GMC growth was inhibited by 50%. [e]TI is the TI and calculated as MHC/GM. Larger values indicate greater cell selectivity. [f]Retention time (t_R) on analytical reverse-phase (RP) HPLC.

hemolytic activity and cytotoxicity. In the presence of 50% TFE, all anoplin analogues have a high α-helical content. Our result shows that the peptides with high antimicrobial activity generally have a high α-helical content in 50% TFE, such as A-C8 (91.01% α-helix, GM = 10.56 μM), K-2A (80.56% α-helix, GM = 8 μM), K-2A-C4 (77.00% α-helix, GM = 6.06 μM), K-2A-C6 (77.51% α-helix, GM = 8 μM), 2K-3A (98.35% α-helix, GM = 4.59 μM), and 2K-3A-C4 (83.97% α-helix, GM = 3.48 μM). However, some new peptides with a high α-helical content in 50% TFE does not exhibit good antimicrobial activity. The α-helical content of these peptides is not directly related to their hemolytic activity and cytotoxicity, as well as the therapeutic index (TI). Therefore, combined with HPLC data, it is speculated that the indiscriminate enhancement in the antimicrobial activity and the toxicity of anoplin analogues after dendrimerization and lipidation may be concerned with the increase in their hydrophobicity, which is only slightly related to the secondary structure of the peptide.

After this screening for antibacterial activity, we assessed the hemolytic potential of these peptides (Table 1, Figures 4, and S1). For this, a hemolytic assay was used in which the leakage of hemoglobin from human red blood cells due to exposure to the dendrimerized and lipidated AMPs was assessed (see the Supporting Information for detailed information on the experiment). Triton X-100 (1%) served as a positive control. Unfortunately, all dendrimeric peptides with high antibacterial activity had high hemolytic activity and a low TI (TI = MHC/GM, where MHC is the minimum hemolytic concentration), for example, the MHC of 2K-3A-C4 is 3.18 μM and TI = 0.91. Also, dendrimerization is more likely to cause hemolysis than lipidation because the cationic nature, hydrophobicity, and molecular volume of the dendrimerized peptides are greatly improved. Thus, the lipidation of a single peptide may be better for the safety of anoplin analogues, such as A-C8, which had a high antibacterial activity (GM = 10.55 μM), a low hemolytic activity (MHC = 210.63 μM), and a high TI = 19.95, which is 20-fold higher than that of 2K-3A-C4 and 2-fold higher than that of anoplin, and had a simpler structure than the dendrimer. The α-helical content of these peptides was not necessarily related to their hemolytic activity. However, the increase in the hydrophobicity of peptides after polymerization and fatty acid modification may increase their hemolytic activity.

Finally, the human glomerular mesangial cell (GMC) line, human renal cortex/proximal tubule epithelial cell line HK-2, and human lung nonsmall cancer cell line A549 were used to determine the cytotoxicity of the peptides by the MTT assay (Table 1, Figures 4, S2, and S3, see the Supporting Information). Similarly, the cytotoxicity of a single peptide after lipidation was

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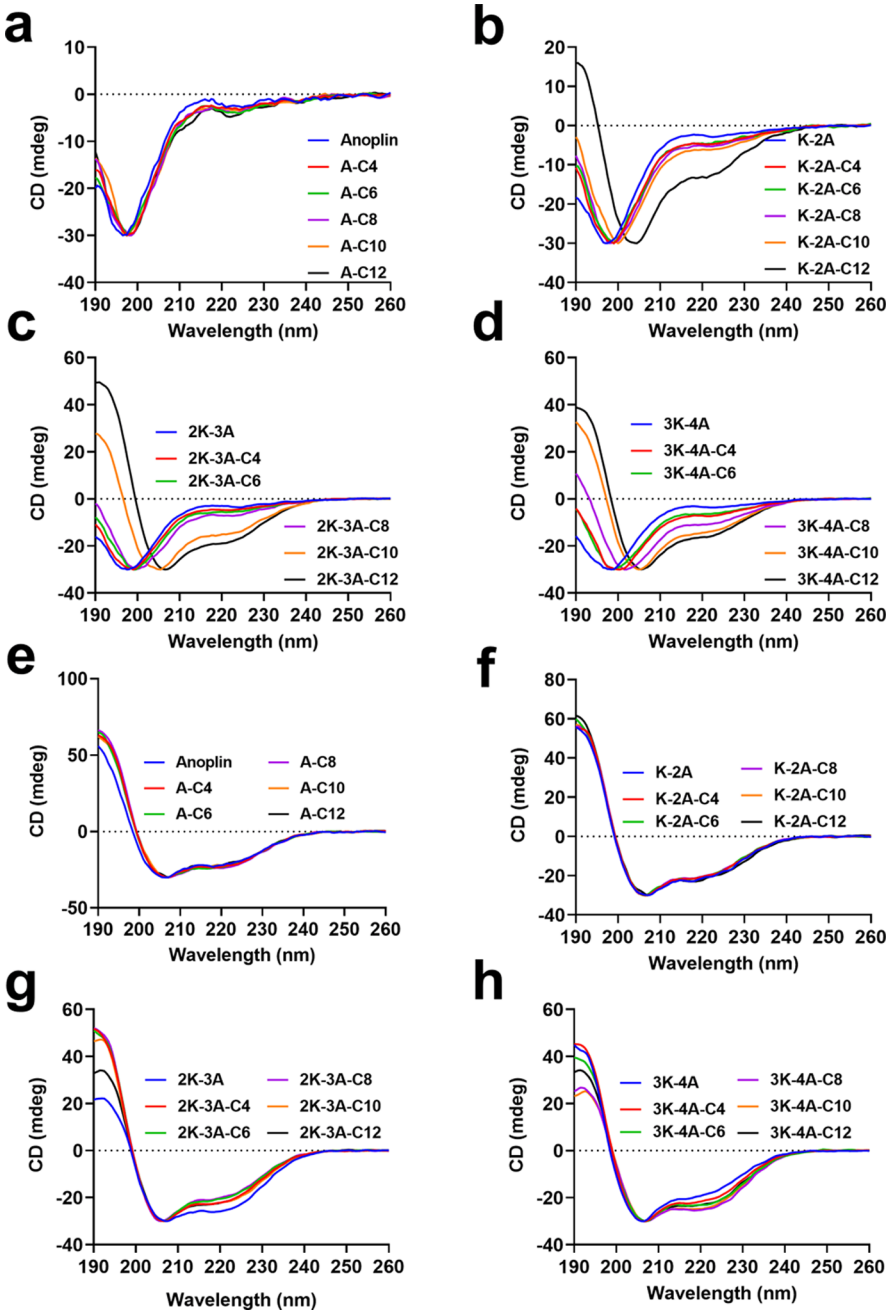

Figure 3. CD spectra of the designed peptides in the presence of water (a–d) and 50% TFE (water as a solvent) (e–h). In water, K-2A-C12, 2K-3A-C10, 2K-3A-C12, 3K-4A-C8, 3K-4A-C10, and 3K-4A-C12 show an α-helical structure. In 50% TFE, all peptides show an α-helical structure.

less than that of the dendrimer after lipidation for these cells tested. In the single peptides (anoplin, A-C4, A-C6, A-C8, A-C10, and A-C12), from anoplin to A-C10, their cytotoxicity increased gradually with an increase in the length of the fatty acid chain, but the cytotoxicity of A-C12 was lower than that of A-C10. In the dimers, from K-2A to K-2A-C8, their cytotoxicity increased gradually with the increase of the length of the fatty acid chain, and from K-2A-C8 to K-2A-C12, their cytotoxicity decreased gradually with the increase of the length of the fatty acid chain. In the trimers, from 2K-3A to 2K-3A-C6, their cytotoxicity increased gradually with the increase of the length of the fatty acid chain, and from K-2A-C6 to K-2A-C12, their cytotoxicity decreased gradually with the increase of the length of the fatty acid chain. There was no specific association between the cytotoxicity of the tetramer and the length of the fatty acid chain. The cytotoxicity of 3K-4a to 3K-4A-C10 are strong, and the cytotoxicity of 3K-4A-C12 was slightly weaker.

For the new peptides that were attached without a fatty acid or with butyric acid or hexanoic acid, the cytotoxicity of the new peptides increased as the number of dendrimer branches (or the degree of dendrimerization) increased. For example, IC_{50} values for GMC of anoplin, K-2A, 2K-3A, and 3K-4A were more than 256, 61.89, 3.73, and 1.69 μM, respectively; IC_{50} values for GMC of A-C4, K-2A-C4, 2K-3A-C4, and 3K-4A-C4 were more than 256, 58.22, 2.05, and 1.28 μM, respectively; and IC_{50} values for GMC of A-C6, K-2A-C6, 2K-3A-C6, and 3K-4A-C6 were more than 256, 42.73, 1.37, and 2.36 μM, respectively.

However, in the new peptides that attached octanoic acid, decanoic acid, and lauric acid, the cytotoxicity of the dimer and tetramer was higher than that of the single peptides and trimer. A

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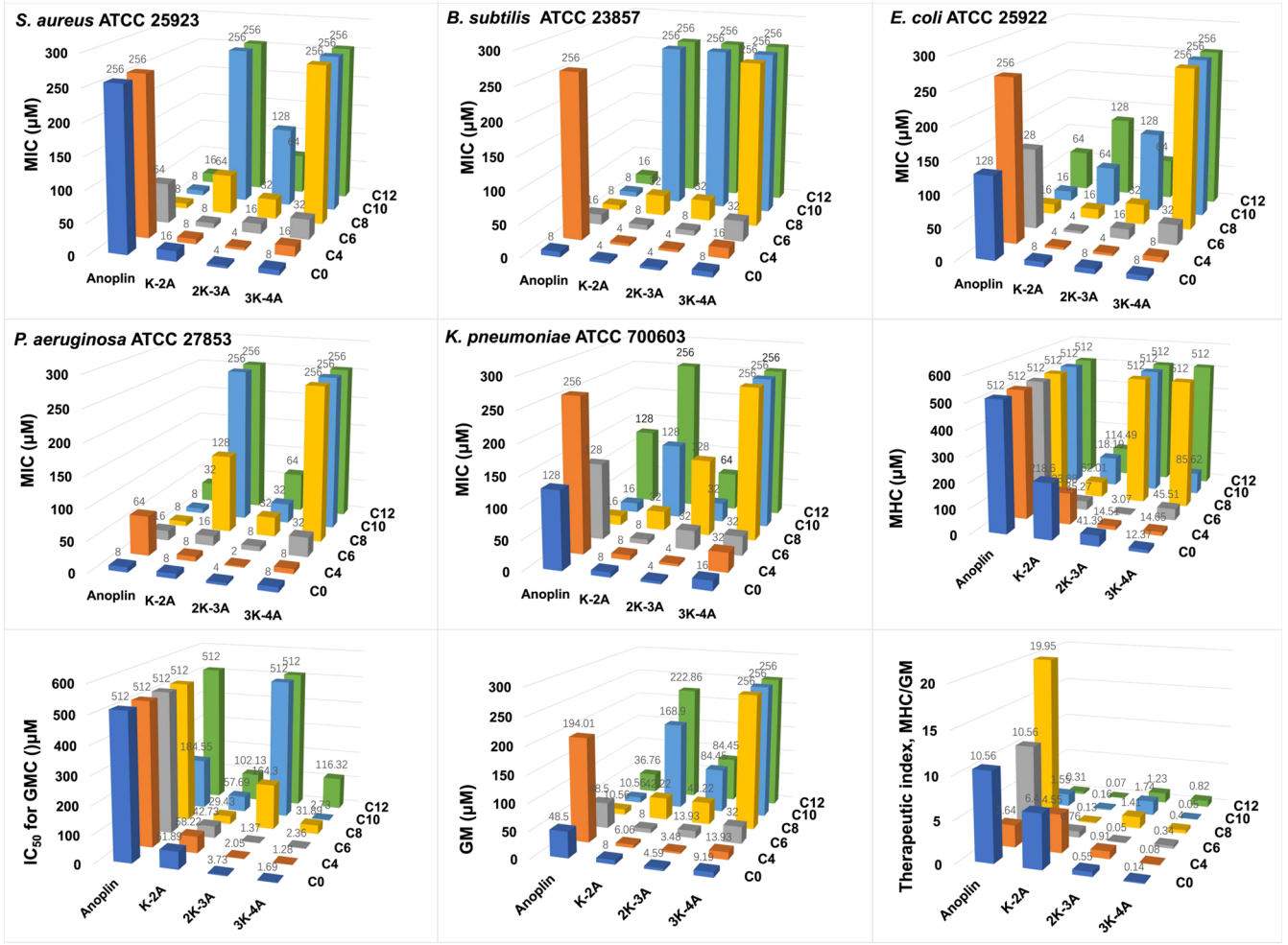

Figure 4. Three-dimensional column plot of the antibacterial activity, hemolytic activity (represented by MHC), cytotoxicity (represented by IC_{50} for GMC), GM of MIC, and TI of anoplin analogues.

Table 2. FIC of the Anoplin Analogues in Combination with Conventional Antibiotics[a]

	P. aeruginosa ATCC 27853		*E. coli* ATCC 25922			*K. pneumoniae* ATCC 700603		
peptide	rifampicin	polymyxin B	rifampicin	polymyxin B	gentamicin	rifampicin	polymyxin B	gentamicin
A-C8	0.5	0.375	0.5625	0.5	0.5	0.3125	0.25	0.5625
A-C10	0.5	0.375	0.5	0.25	0.5	0.375	0.3125	0.75
K-2A	0.375	0.3125	0.25	0.125	0.3125	0.5625	0.5	0.625
K-2A-C4	0.5	0.37	0.3125	0.25	0.5	0.375	0.3125	0.625
K-2A-C6	0.375	0.25	0.25	0.125	0.375	0.25	0.125	0.375
2K-3A	0.5	0.375	0.3125	0.25	0.5625	0.375	0.3125	0.625
2K-3A-C4	0.5	0.375	0.3125	0.25	0.5625	0.375	0.3125	0.625
2K-3A-C6	0.375	0.25	0.25	0.125	0.5	0.125	0.125	0.375
3K-4A	0.25	0.25	0.25	0.125	0.3125	0.25	0.125	0.375
3K-4A-C4	0.25	0.1875	0.125	0.125	0.25	0.25	0.125	0.375
anoplin	0.3125	0.25	0.125	0.125	0.25	0.125	0.125	0.5
polymyxin B	0.3125	ND	0.125	ND	0.3125	0.3125	ND	0.5

[a]Fractional inhibitory concentration index (FIC) calculated using a checkerboard is defined according to the following equation: FIC = (MIC of peptide in combination)/(MIC of peptide alone) + (MIC of antibiotic in combination)/(MIC of antibiotic alone). FIC was interpreted as follows: FIC ≤0.5: synergy; 0.5 < FIC < 1: additive effect; 1.0 ≤ FIC < 4.0: indifference; and FIC ≥ 4: antagonism. We only selected the more active analogues along with antibiotics. "ND" means no detection.

special phenomenon is that 3K-4A-C10 had much high cytotoxicity than other analogues modified with decanoic acid (A-C10, K-2A-C10, and 2K-3A-C10). The results show that the cytotoxicity of the dendrimerized analogues modified without fatty acids or with short-chain fatty acids (C4 and C6) was higher than those modified with long-chain fatty acids (C8–C12), and the cytotoxicity of the new peptides will be increased with the increase of the number of branches of these dendrimers.

According to our previous research, the polymerized peptide had higher antibacterial activity and stability than a single

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peptide, and the toxicity was almost unchanged.[10] However, in this study, the activity of the polymerized peptides was significantly improved, and their toxicity was greatly improved. All polymeric peptides are not ideal candidates for AMPs due to their strong toxicity. According to reports,[17] AMPs have two main mechanisms of action to the bacterial membranes, namely, the carpet-like mechanism of action attached to the surface of the bacterial membrane and the perforation mechanism or the barrel mechanism across the bacterial membrane. The single peptide has a linear structure, and the polymerized peptide has a bulky multibranched three-dimensional structure. The mechanism of action of A-C8 on the bacterial membrane is a transmembrane mechanism. However, the polymerized peptides may have only the mechanism of covering the surface of the bacterial membrane due to their large size. Mammalian cell membranes contain cholesterol and are more rigid than bacterial membranes. Therefore, A-C8 can cross the bacterial membranes but not the mammalian cell membranes, showing high cell selectivity. The polymerized peptide can cover the surface of the bacterial membrane and mammalian cell membrane indiscriminately, showing low selectivity. Thus, we would rather choose A-C8 than other peptides.

Because the peptides showed moderate activity against resistant strains, these peptides combined with certain conventional antibiotics. The new peptides with GM < 16 were used in combination with the conventional antibiotics (results are shown in Table 2), with polymyxin B serving as a control. These new peptides in combination with the conventional antibiotics have shown synergistic or additive effects. It is interesting that a conventional antibiotic along with a low antimicrobial active peptide has a better synergistic effect than a conventional antibiotic along with a high antimicrobial active peptide. The synergistic effect of these new peptides along with polymyxin B is better than that along with other conventional antibiotics. The synergistic effect of conventional antibiotics along with polymyxin B is generally better than that along with the new peptides.

CONCLUSIONS

In conclusion, in the development of AMPs, excessive chemical modifications may not be necessary for some peptides. In contrast, simple modifications may lead to unexpected and satisfactory results.[18−20] The direct lipidation of a single anoplin is better than the lipidation after dendrimerization for antibacterial performance and cell selectivity. Dendrimerization increases the hydrophobicity, cationic nature, α-helicity, and multi-valency of these compounds, thereby increasing the antibacterial activity. However, for cell selectivity or biocompatibility, an excessive increase in the hydrophobicity, cationic nature, and multivalency is not necessary because hemolytic activity and toxicity are also increased. Although the trimer 2K-3A-C4 has a satisfactory antibacterial activity, its cell selectivity is too low. Taken together, A-C8 is a satisfactory candidate AMP, which will be useful for the future development of peptide-based antibiotic candidates.

EXPERIMENTAL SECTION

Synthesis and Characterization of Peptides. Peptides were synthesized using Rink Amide MBHA resin and Fmoc based on solid-phase peptide synthesis. The peptide extension reaction conditions were as follows: DMF served as a solvent, the Fmoc-protected group was removed using 20% piperidine solution (in DMF, 2−5 min × 4), the Mtt-protecting group was removed using 1% trifluoroacetic acid solution (in dichloromethane, 2 min × 10−15), a 3-fold excess of HBTU served as the coupling reagent, 3-fold excess of HOBt served as the additive, 6-fold excess of DIPEA served as the base, and 3-fold excess of Fmoc-protected amino acids. Peptide cleavage from the resin was performed using a reagent composed of 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% deionized water. The peptide was precipitated in cold diethyl ether. The obtained crude peptides were purified by RP-HPLC (Waters, MA, USA) on a C18 column (19 × 250 mm). These peptides were analyzed using RP-HPLC on a C18 column (4.6 × 250 mm). All peptides prepared had a purity of more than 95%. Their molecular masses were confirmed via ESI-MS (MaXis 4G, Bruker, USA).

Circular Dichroism. The CD spectra of the peptides were measured at 25 °C on a J-810 spectrometer (Jasco, Japan) using a quartz cell with a 1.0 mm path length. Recording was performed over 190−260 nm at a scanning speed of 50 nm/min, a bandwidth of 1 nm, and a response time of 1 s. The peptide solutions were prepared at a final concentration of 50 μM in deionized water (mimicking an aqueous environment) and 50% TFE (mimicking the hydrophobic environment of the biological membrane). The acquired CD spectra were converted to the mean residue ellipticity using the following equation

$$\theta_M = (\theta_{obs} \times 1000)/(c \times l \times n)$$

where θ_M is the residue ellipticity (deg·cm^2·dmol^{-1}), θ_{obs} is the observed ellipticity corrected for the buffer at a given wavelength (mdeg), c is the peptide concentration (μM), l is the path length (mm), and n is the number of amino acids.

Determination of MICs. The MICs of the peptides were determined using the Clinical and Laboratory Standards Institute broth dilution procedure. Briefly, a single colony of the bacterial strain was transferred to 4 mL of MHB and cultured at 37 °C. After 16−24 h, bacterial culture in the logarithmic phase was diluted to 1×10^6 CFU/mL. The assay was performed by adding 50 μL of each peptide solution at various concentrations and 50 μL of diluted bacterial culture to different wells of a 96-well microtiter plate. After incubation at 37 °C for 18 h, the lowest concentration of the peptides with no obvious bacterial growth was identified as the MIC. At least three independent experiments were conducted for the MIC assays, and three technical replicates were used in each independent experiment.

Biocompatibility Assays. Human GMC, HK-2, and A549 cell lines were used to determine the cytotoxicity of the peptides using the MTT assay. Healthy mouse erythrocytes were used to evaluate the hemolytic rates of the peptides using a previously described procedure. Briefly, for the MTT assay, cells were inoculated into 96-well plates at a density of 1.0×10^4 cells/well and were interacted with various peptides (1−256 μM) for 12 h at 37 °C and 5% CO_2. Subsequently, the cell culture was further incubated with 0.5 mg/mL MTT for 4 h at 37 °C. Then, the supernatant was discarded, and formazan crystals were dissolved in 150 μL of dimethyl sulfoxide. The solution was further measured using a microplate reader (Molecular Devices FlexStation III, USA) at an optical density of 570 nm.

The venous blood of male BALB/C mice weighing 20 ± 2 g was collected from the venous plexus of the fundus and centrifuged at 1000×g for 10 min. The plasma supernatant was removed, and 8% erythrocyte suspension was prepared using phosphate-buffered saline (PBS; pH = 7.4). Then, 100 μL of 8%

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erythrocyte suspension and 100 μL of peptides (0, 1, 2, 4, 8, 16, 32, 64, 128, or 256 μM) in PBS were added to the 96-well plates, and 2% Triton X-100 was used as a positive control. The plates were incubated at 37 °C for 1 h and centrifuged at 12,000*g* for 15 min. Plasma supernatants (150 μL) were transferred to new 96-well plates. Absorbance was measured using a multimode reader at 490 nm. The peptide concentration that caused a hemolytic rate of more than 10% was considered the minimal hemolysis concentration. At least three independent experiments were conducted for the biocompatibility assays, and three technical replicates were used in each independent experiment.

Combination with Conventional Antibiotics. Peptides with high antibacterial activity used along with conventional antibiotics against *E. coli* 25922, *P. aeruginosa* 27853, and *K. pneumoniae* 700603 were investigated by determining the FIC index using the checkerboard assay, as previously described with minor modifications.[21] The peptides and antibiotics were mixed at their 2× MIC, and then 2-fold serial dilutions were prepared in the MHB medium. An equal volume of the above mixtures and bacteria (1×10^6 CFU mL^{-1}) was added to 96-well plates and incubated for 18−24 h at 37 °C. The next steps were consistent with the MIC determination methods. FIC was defined according to the following equation

$$\begin{aligned} \text{FIC} = &(\text{MIC of peptide in combination}) \\ &/(\text{MIC of peptide alone}) \\ &+ (\text{MIC of antibiotic in combination}) \\ &/(\text{MIC of antibiotic alone}) \end{aligned}$$

FIC was interpreted as follows: FIC ≤ 0.5: synergy; 0.5 < FIC <1: additive effect; 1.0 ≤ FIC < 4.0: indifference; and FIC ≥ 4: antagonism.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c01854.

Hemolytic activity and cytotoxicity of all anoplin analogues and ESI mass spectra and analytical RP-HPLC chromatograms of purified peptides (PDF)

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Author Contributions

S.G. and J.N. designed and conceived the experiments. S.G. and B.L. conducted the main experiments assay. X.Y. and Z.B. conducted the in vivo assay. C.Z. conducted the SEM and LSCM assay. S.G. wrote the main manuscript text. J.N. supervised the work and revised the final version of the manuscript. All the authors have read and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AMP, antimicrobial peptide; GM, geometric mean; GMC, human glomerular messangial cell; MIC, minimum inhibitory concentration; MS, mass spectrometry; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide; RP-HPLC, reverse-phase high-performance liquid chromatography; TI, therapeutic index

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