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Article

Rational Avoidance of Protease Cleavage Sites and Symmetrical End-Tagging Significantly Enhances the Stability and Therapeutic Potential of Antimicrobial Peptides

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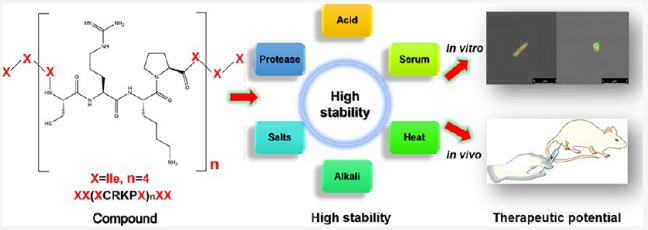
ABSTRACT: Antimicrobial peptides (AMPs) are expected to solve problem of antibiotic resistance because of their distinctive nonspecific membrane-disruptive mechanism. However, clinical applications of AMPs have been precluded by their poor stability, although various complex chemical strategies have been employed to solve this problem, and this undoubtedly greatly increases the manufacturing cost. Herein, a series of novel peptides with high stability were developed based on protease-specific cleavage sites and symmetrical end-tagging. Among these peptides, II-I_4-II exhibited the best antibacterial activity and the highest therapeutic index. More importantly, II-I_4-II showed extremely high stability in the presence of various proteases, physiological salts and serum, and under acid, alkali, and heat conditions, and it exhibited excellent therapeutic potential *in vivo*. Additionally, II-I_4-II exhibited a membrane-disruptive mechanism and a low propensity to induce resistance. In general, these findings contribute to the design of AMPs with high stability and might accelerate clinical applications of AMPs.



INTRODUCTION

In the past 90 years, antibiotics have saved countless lives and enabled key medical procedures, including surgery and cancer chemotherapy,[1] which was hailed as one of the most important medical achievements of the 20th century.[2] However, it is clear that overuse of antibiotics has accelerated the development of resistance in pathogens.[3] It is assessed that 700 thousands people die globally per year from diseases caused by antibiotic resistance, and worse still, the number could increase to 10 million by 2050 if more effective antibiotics are not developed.[4] Thus, discovering novel antibacterial agents, especially those with new mechanisms of action, has become a top priority.

Antimicrobial peptides (AMPs), are widely present in natural organisms as an important part of the host immune system and have a variety of biological activities,[5,6] such as antibacterial,[7] antifungal,[8] and anti-inflammatory activity.[9] Although the precise bactericidal mechanism of AMPs has not yet been fully understood and is still controversial, there is no doubt that most AMPs kill bacteria by destroying the integrity of bacterial membranes.[10] This unique nonspecific membrane-disruptive mechanism can effectively curb the development of bacterial resistance.[11] Therefore, AMPs are considered to be the most promising antimicrobial agents as an alternative to antibiotics.[12,13] However, clinical application of AMPs has been restricted by poor stability; for example, they are easily degraded by proteases (among which, trypsin, chymotrypsin, and pepsin have been deemed the biggest menaces to AMPs) and can be inactivated in the presence of physiological salts or serum.[14−16] According to the specificity of proteolysis, trypsin preferentially cleaves the peptide bond on the C-terminal side of the cationic residue (Arg and Lys). Conversely, chymotrypsin and pepsin preferentially cleave the peptide bond on the C-terminal side of the hydrophobic residue (Phe, Leu, Tyr, and Trp), which makes it possible that AMPs containing a combination of cationic residue and hydrophobic residue sequences may be hydrolyzed by trypsin, chymotrypsin, or pepsin.[17,18] For the past few years, in order to improve the stability of AMPs, numerous chemical modification strategies have been proposed, including peptidomimetic, multimeric peptides, cyclization, and the use of amino acid analogues or unnatural amino acids.[19−22] Disappointingly, although these complex chemical modifications have raised the stability of AMPs to some extent, the resulting poor biocompatibility and high manufacturing costs have further hindered their clinical application.[18,23] Therefore, new strategies must be developed to improve the stability of AMPs without

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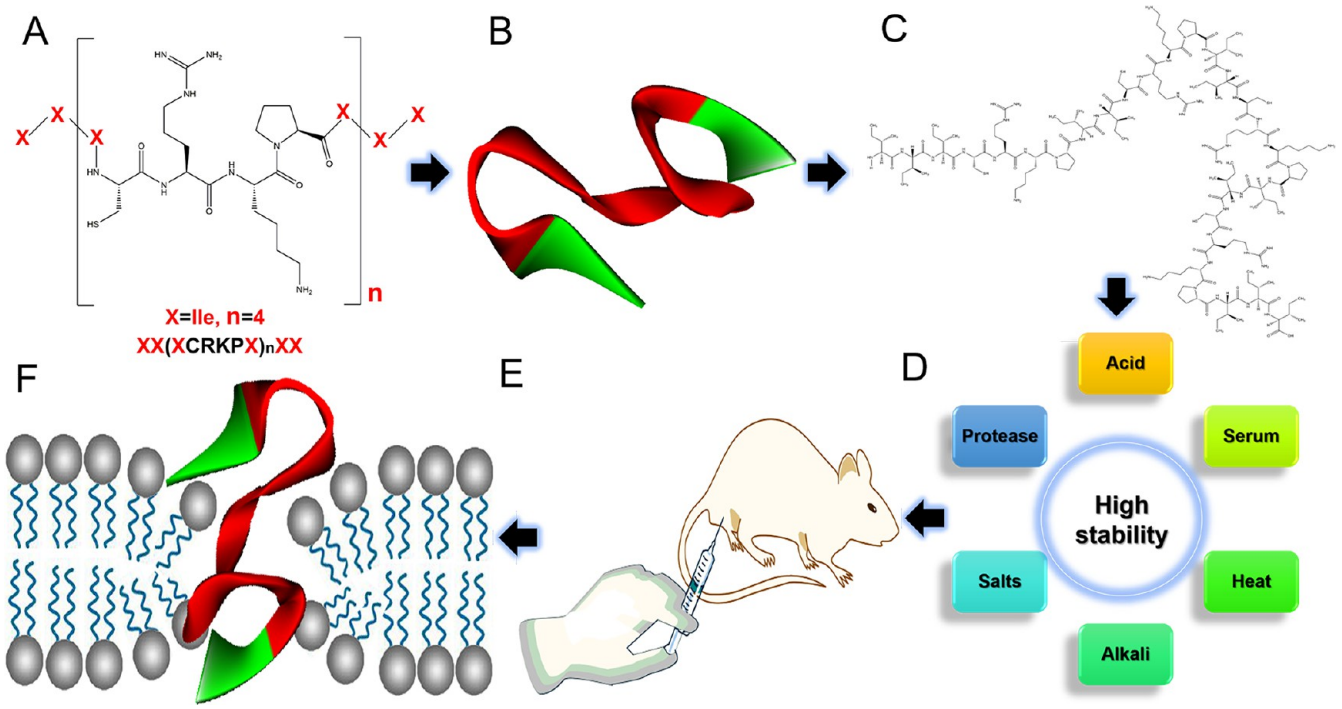

Figure 1. (A) Structural formula and sequence of the II-I_4-II. (B) Three-dimensional structure of the II-I_4-II was predicted online by I-TASSER.[34] (C) Schematic chemistry structure of the II-I_4-II. (D) Schematic diagram of II-I_4-II with high stability. (E) Therapeutic potential of II-I_4-II *in vivo*. (F) Schematic model of the interaction of the II-I_4-II with the bacterial membranes.

Table 1. Peptide Sequence and Its Main Physical and Chemical Parameters

peptide	sequence	theoretical MW (Da)[a]	measured MW (Da)	purity (%)	net charge	H^b
V_2	VCRKPVVCRKPV-NH_2	1382.79	1382.78	99.260	5	0.450
V_3	VCRKPVVCRKPVVCRKPV-NH_2	2065.68	2065.66	98.469	7	0.450
V_4	VCRKPVVCRKPVVCRKPVVCRKPV-NH_2	2748.56	2748.54	95.085	9	0.450
V_5	VCRKPVVCRKPVVCRKPVVCRKPVVCRKPV-NH_2	3431.44	3431.45	97.163	11	0.450
I_2	ICRKPIICRKPI-NH_2	1438.90	1438.88	99.628	5	0.643
I_3	ICRKPIICRKPIICRKPI-NH_2	2149.84	2149.85	99.245	7	0.643
I_4	ICRKPIICRKPIICRKPIICRKPI-NH_2	2860.77	2860.78	98.996	9	0.643
I_5	ICRKPIICRKPIICRKPIICRKPIICRKPI-NH_2	3571.71	3571.68	97.759	11	0.643
I-I_2-I	IICRKPIICRKPII-NH_2	1665.22	1665.20	99.292	5	0.809
I-I_3-I	IICRKPIICRKPIICRKPII-NH_2	2376.15	2376.13	97.322	7	0.759
I-I_4-I	IICRKPIICRKPIICRKPIICRKPII-NH_2	3087.09	3087.07	95.840	9	0.732
I-I_5-I	IICRKPIICRKPIICRKPIICRKPIICRKPII-NH_2	3798.02	3798.00	99.621	11	0.716
II-I_2-II	IIICRKPIICRKPIII-NH_2	1891.53	1891.52	98.483	5	0.993
II-I_3-II	IIICRKPIICRKPIICRKPIII-NH_2	2602.47	2602.45	99.233	7	0.854
II-I_4-II	IIICRKPIICRKPIICRKPIICRKPIII-NH_2	3313.40	3313.39	96.956	9	0.809
II-I_5-II	IIICRKPIICRKPIICRKPIICRKPIICRKPIII-NH_2	4024.34	4024.32	96.995	11	0.779

[a]The theoretical MW of the peptide was analyzed using the ProtParam tool.[28] [b]The mean hydrophobicity (H) of the peptide was analyzed using HeliQuest (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py).

complex chemical modifications and expensive unnatural amino acids.

In the present study, we attempted to improve the stability of AMPs through systematic formulation of natural amino acids. Previous studies have shown that the net positive charge and hydrophobicity are the main traits of AMPs and also one of the decisive factors affecting their antibacterial activity.[13,24] The net positive charge is conscientious for selective targeting to negatively charged components of bacterial membranes, and hydrophobicity plays an important role in regulating membrane interactions.[25−27] Thus, we chose Arg as cationic amino acids to provide the necessary net positive charge, and Cys and Lys were placed at the N-terminal side and C-terminal side of Arg, respectively, to block cleavage of Arg by trypsin. Simultaneously, Pro was placed at the C-terminal side of Lys to block cleavage of Lys by trypsin.[17,28] To avoid cleavage by chymotrypsin and pepsin, Ile or Val with long aliphatic side chains were selected as hydrophobic amino acids to be placed at the N-terminal side of Cys and C-terminal side of Pro to provide the necessary hydrophobicity and increase the depth of the polar surface. Meanwhile, symmetrical end-tagging of peptides with hydrophobic amino acids was used to improve antibacterial activity and stability.[29−32] Thus, the resulting sequence template with high stability was XX(XCRKPX)$_n$XX (where n = 2, 3, 4, or 5; X = I or V) (Figure 1), and the C-terminal of sequence was aminated to maintain high stability (Table 1).[33] All peptides were first

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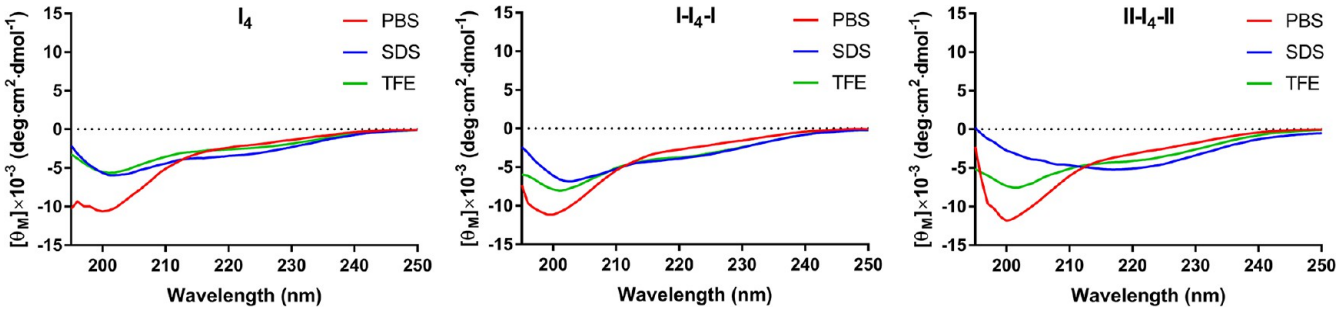

Figure 2. CD spectra of the I_4, I-I_4-I and II-I_4-II.

Table 2. MICs[a] (μM) of the Peptides

	Gram-negative bacteria						Gram-positive bacteria					
peptide	*E. coli* 25922	*E. coli* UB1005	*S. typhimurium* 14028	*S. typhimurium* 7731	*S. choleraesuis* 503	*S. pullorum* 7913	*S. aureus* 29213	*S. aureus*[b] 11011	MRSA[c] 43300	*E. faecalis* 29212	*S. epidermidis* 12228	*S. agalactiae*[d] H92
V_2	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
V_3	8	16	8	16	>64	>64	>64	>64	>64	>64	>64	>64
V_4	8	8	8	8	>64	64	>64	>64	>64	>64	>64	>64
V_5	4	4	4	4	>64	16	>64	>64	>64	>64	>64	>64
I_2	64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
I_3	8	4	8	8	16	64	>64	>64	>64	>64	>64	>64
I_4	2	2	2	4	8	16	>64	>64	>64	>64	>64	>64
I_5	4	2	4	4	8	8	>64	>64	>64	>64	>64	>64
I-I_2-I	16	16	32	32	>64	>64	>64	>64	>64	>64	>64	>64
I-I_3-I	4	4	8	8	16	32	>64	>64	64	>64	64	64
I-I_4-I	2	1	2	2	4	8	32	16	16	32	16	16
I-I_5-I	2	2	4	2	4	8	>64	>64	64	>64	64	>64
II-I_2-II	8	8	32	32	32	32	>64	>64	>64	>64	>64	64
II-I_3-II	4	4	8	8	16	16	>64	>64	>64	>64	>64	16
II-I_4-II	2	2	2	2	2	4	8	4	4	8	4	2
II-I_5-II	4	2	4	4	4	8	>64	>64	>64	>64	>64	>64
melittin	2	2	2	2	4	4	2	2	2	2	2	1

[a]The values of MICs are representative of three independent tests. [b]*S. aureus* 11011 is a clinical isolate. [c]Methicillin-resistant *S. aureus*. [d]*S. agalactiae* H92 is a clinical isolate.

assessed for their antimicrobial activity and biocompatibility, and the stability of peptides with a high therapeutic index (TI) was evaluated under various conditions. Then, the development of resistance and the *in vivo* therapeutic potential of the target peptide were evaluated. In the end, the target peptide mechanism was preliminarily studied through a variety of membrane permeabilization analyses and electron microscopy.

RESULTS AND DISCUSSION

Design and Characterization of the Peptides. In this research, we sought to design a template sequence XX-$(XCRKPX)_nXX$ with high stability based on protease-specific cleavage sites and symmetrical end-tagging. Primarily, we selected Val as a hydrophobic amino acid in the $(XCRKPX)_n$ series to build peptides with different numbers of repeat units. Second, Val was replaced with Ile, which has stronger hydrophobicity to explore whether the antimicrobial activity of the peptides could be increased without causing toxicity. Finally, symmetrical end-tagging of the peptides with Ile was performed in a try to improve antibacterial activity and stability. The sequences and main physical and chemical parameters of the peptides are listed in Table 1 and Figure S1. The purity and molecular weight (MW) of the peptides were determined by reversed-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (MS), respectively, after the peptides were synthesized. The results showed that the measured MW was consistent with the theoretical MW, and the purity was >95%, indicating that the synthesized peptides were consistent with the designed peptides. The net positive charge of the novel peptides ranged from 5 to 11. The novel peptides with 2, 3, 4, and 5 repeat units had net positive charges of 5, 7, 9, and 11, respectively. As the C-terminal and the N-terminal of peptides were symmetrically end-tagged with hydrophobic amino acids, the mean hydrophobicity of the peptides increased and followed the order $XX(XCRKPX)_nXX$ > $X(XCRKPX)_nX$ > $(XCRKPX)_n$. Moreover, the secondary structure of the peptides was inspected by circular dichroism (CD) (Figures 2 and S2). In 10 mM phosphate buffered saline (PBS), most peptides exhibited a disordered structure. In 50% 2,2,2-trifluoroethanol (TFE) and 30 mM sodium dodecyl sulfate (SDS), most peptides exhibited a strong negative peak close to 203 nm and a weak negative peak close to 222 nm, indicating the presence of a partial α-helical structure in those peptides,[35] possibly due to the presence of proline, which is a potent α-helix breaker.[36,37]

Antimicrobial Activity. The antimicrobial activity of the peptides against 12 species of bacteria (including one drug-resistant bacteria and two clinical isolates) is presented in Table

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Table 3. MHC, GM, and TI Values of the Peptides

		GM (μM)[b]			TI[c]		
peptide	MHC (μM)[a]	Gram-negative bacteria	Gram-positive bacteria	all	Gram-negative bacteria	Gram-positive bacteria	all
V_2	>128	64.00	64.00	64.00	2.00	2.00	2.00
V_3	>128	20.16	64.00	35.92	6.35	2.00	3.56
V_4	>128	16.00	64.00	32.00	8.00	2.00	4.00
V_5	>128	8.00	64.00	22.63	16.00	2.00	5.66
I_2	>128	64.00	64.00	64.00	2.00	2.00	2.00
I_3	>128	11.31	64.00	26.91	11.31	2.00	4.76
I_4	>128	4.00	64.00	16.00	32.00	2.00	8.00
I_5	>128	4.49	64.00	16.95	28.51	2.00	7.55
I-I_2-I	>128	32.00	64.00	45.25	4.00	2.00	2.83
I-I_3-I	>128	8.98	64.00	23.97	14.25	2.00	5.34
I-I_4-I	>128	2.52	20.16	7.13	50.80	6.35	17.96
I-I_5-I	>128	3.17	64.00	14.25	40.32	2.00	8.98
II-I_2-II	>128	20.16	64.00	35.92	6.35	2.00	3.56
II-I_3-II	>128	8.00	50.80	20.16	16.00	2.52	6.35
II-I_4-II	>128	2.24	4.49	3.17	57.02	28.51	40.32
II-I_5-II	>128	4.00	64.00	16.00	32.00	2.00	8.00
melittin	1	2.52	1.78	2.12	0.40	0.56	0.47

[a]The MHC is the minimum peptide concentration that caused >5% hemolysis of human red blood cells (hRBCs). When no detectable hemolytic activity was observed at 128 μM, a value of 128 μM was used to calculate the TI. [b]The GM of the MIC values of the peptides against measured bacteria was calculated. When no detectable antimicrobial activity was observed at 64 μM, a value of 64 μM was used to calculate the TI. [c]The TI was calculated as MHC/GM. Lager values indicate higher therapeutic potential.

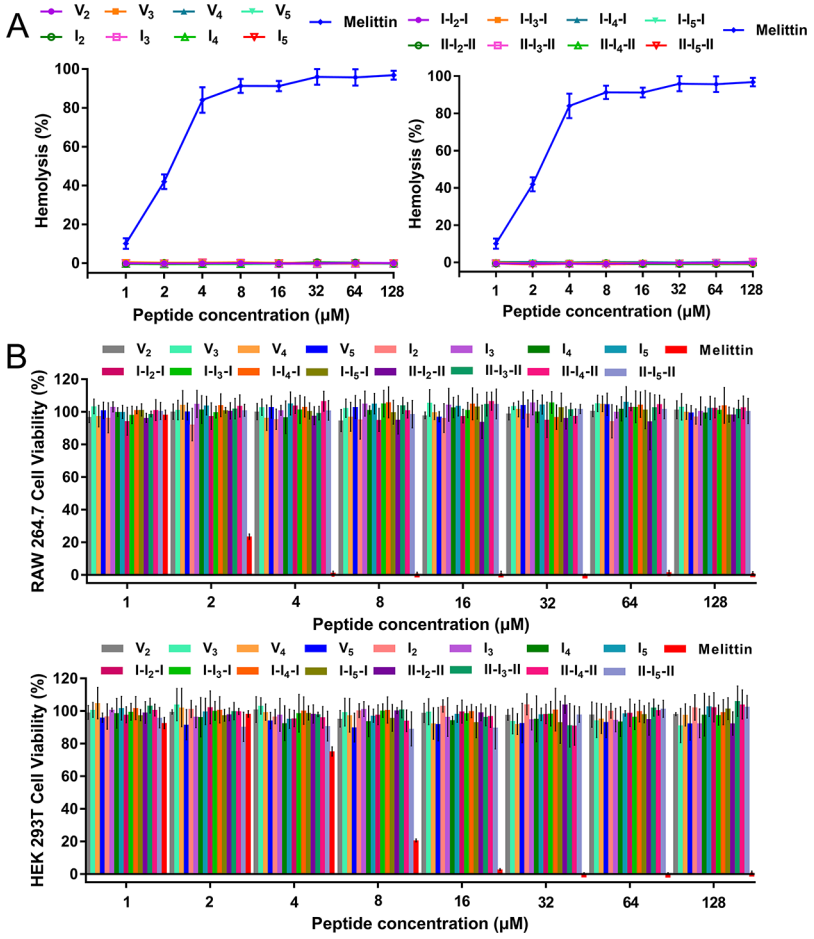

Figure 3. Hemolytic activity and cytotoxicity of the peptides. (A) Hemolytic activity of the peptides against hRBCs. (B) Cytotoxicity of the peptides against RAW 264.7 cells and HEK 293T cells.

2. Additionally, the geometric mean (GM) of the minimum inhibitory concentration (MIC) values of the peptides is shown in Table 3. For the $(VCRKPV)_n$ series, the antimicrobial activity of the peptides against Gram-negative bacteria tended to be

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J. Med. Chem. 2020, 63, 9421−9435

Table 4. Physiological Salts and Serum Stability of Peptides

peptide	control	physiological salts						GM[a]	fold[b]	serum			GM[a]	fold[b]
		Na^+	K^+	Mg^{2+}	Zn^{2+}	NH_4^+	Fe^{3+}			25%	50%	100%		
E. coli 25922														
I_4	2	32	4	8	2	2	4	5.04	2.52	4	8	16	8.00	4.00
I-I_4-I	2	8	2	8	2	2	2	3.17	1.59	4	4	16	6.35	3.17
II-I_4-II	2	4	2	4	2	2	2	2.52	1.26	4	4	8	5.04	2.52
melittin	2	4	2	4	2	2	2	2.52	1.26	8	8	8	8.00	4.00
S. aureus 29213														
I_4	>64	>64	>64	>64	>64	>64	>64	N.D[c]	N.D[c]	>64	>64	>64	N.D[c]	N.D[c]
I-I_4-I	32	64	64	64	32	32	32	45.25	1.41	64	64	64	64.00	2.00
II-I_4-II	8	16	8	16	8	8	8	10.08	1.26	8	16	32	16.00	2.00
melittin	2	4	2	4	2	2	2	2.52	1.26	8	4	8	6.35	3.17

[a]GM of MIC values. [b]Fold change of MIC values (GM/control). [c]No data.

enhanced as the number of repeat units increased. Because these peptides have the same the mean hydrophobicity, the net charge may be the main factor affecting their antimicrobial activity. It is generally assumed that the interaction between AMPs and the bacterial membranes primarily depends on electrostatic attraction,[38] while cationic amino acids are the crucial driving force,[39] and thus, an increase in net charge within a certain range generally leads to an improvement in antimicrobial activity. For the $(ICRKPI)_n$ series, the antimicrobial activity of $(ICRKPI)_n$ series repeat units was better than that of $(VCRKPV)_n$, which may be due to the improvement in mean hydrophobicity. Many studies have confirmed that an increase in the hydrophobicity of AMPs within a certain range can enhance their antimicrobial activity because the hydrophobic amino acids in AMPs can be inserted into the hydrophobic region of the membrane, causing the membrane to rupture and ultimately leading to death of the bacteria.[26,40] However, compared with I_4 ($GM_{Gram\text{-}negative\ bacteria}$ = 4.00), I_5 ($GM_{Gram\text{-}negative\ bacteria}$ = 4.49) showed no further enhancement in antimicrobial activity, which was similar to the previous results.[41] Most AMPs have a net positive charge ranging from 2 to 9, and an excess net positive charge may not increase or could even decrease antimicrobial activity.[13] For the $X(XCRKPX)_nX$ and $XX(XCRKPX)_nXX$ series repeat units, I-I_4-I ($GM_{Gram\text{-}negative\ bacteria}$ = 2.52) and II-I_4-II ($GM_{Gram\text{-}negative\ bacteria}$ = 2.24) showed the best activity against Gram-negative bacteria. Interestingly, I-I_4-I ($GM_{Gram\text{-}positive\ bacteria}$ = 20.16) exhibited weak activity against Gram-positive bacteria, while II-I_4-II ($GM_{Gram\text{-}positive\ bacteria}$ = 4.49) showed satisfactory activity against Gram-positive bacteria. In short, II-I_4-II (GM_{All} = 3.17) displayed the best activity for all bacteria, comparable to that of melittin (GM_{All} = 2.12). The above results show that the sequence length, net positive charge, and hydrophobicity of AMPs are closely intertwined to the antimicrobial activity.

Hemolytic Activity and Cytotoxicity. High hemolysis and cytotoxicity of the AMPs are factors that have limited clinical translation. The hemolytic activity of the peptides is depicted in Figure 3A, and the minimum hemolytic concentration (MHC) is calculated and summarized in Table 3. All the designed peptides showed <5% hemolysis at concentrations ranging from 1 to 128 μM. On the contrary, melittin induced 10.05% hemolysis at a concentration of only 1 μM, which showed extremely high hemolytic activity. As shown in Figure 3B, on the one hand, all the designed peptides showed negligible cytotoxicity toward the mouse macrophage cell line RAW 264.7 and the human embryonic kidney (HEK) 293T cells, with cell viabilities greater than 90% at a concentration of 128 μM. On the other hand, melittin induced a large number of RAW 264.7 and HEK 293T cells death at only a lower concentration.

Furthermore, based on the antimicrobial activity and hemolytic activity, the TI was calculated based on the ratio of MHC to GM, which is an important index in evaluating the clinical application value of peptides (Table 3). Among all the designed peptides, II-I_4-II (TI_{All} = 40.32) exhibited the highest TI. This selectivity might be due to differences in the composition of bacterial cell membranes and mammalian cell membranes.[42] Compared with mammalian cell membranes which have zwitterionic phosphatidylcholines, the proportion of negatively charged phospholipids on the outside of bacterial cell membranes is significantly higher; therefore, AMPs can target bacterial membranes though electrostatic attraction.[43] Moreover, for Gram-negative bacteria, I_4 ($TI_{Gram\text{-}negative\ bacteria}$ = 32.00) and I-I_4-I ($TI_{Gram\text{-}negative\ bacteria}$ = 50.80) also exhibited a satisfactory TI value. In contrast, as a control peptide, melittin (TI_{All} = 0.47) had a very low TI value because of its extremely high hemolytic activity, which was similar to the previous results.[39,41]

Salts, Serum, Acid, Alkali, and Heat Stability. Based on the above analysis results, I_4, I-I_4-I and II-I_4-II were selected for stability research. Complex physiological environments can limit the clinical applications of AMPs *in vivo*. Therefore, the stability of peptides in different environments was assessed by measuring changes in the MIC value against *E. coli* 25922 and *S. aureus* 29213 (Table 4 and S1).

Although AMPs have powerful antimicrobial activity *in vitro*, it is partially or completely lost in the presence of physiological salts.[18] In physiological salts, the antimicrobial activity of all peptides was nearly constant in the presence of K^+, Zn^{2+}, NH_4^+, and Fe^{3+}, but the presence of Na^+ and Mg^{2+} caused a decrease in the antimicrobial activity of all peptides (Table 4). Previous studies have shown that Na^+ can obstruct the electrostatic attraction between peptides and membranes though the charge screening effect.[18] Mg^{2+} can reduce the binding efficiency of peptides to membranes by binding to anionic phosphate groups.[44] In this study, we found that the salt stability increased when the C-terminal and N-terminal of the peptide were end-tagged with hydrophobic amino acids and followed the order II-I_4-II > I-I_4-I > I_4. As everyone knows that the salt stability of AMPs is closely corresponded to net positive charge and hydrophobicity.[18] Thus, because I_4, I-I_4-I and II-I_4-II have the same net positive charge, we believe that the above results may be due to the different hydrophobicity. End-tagging with the hydrophobic group may help the peptide penetrate deeper into

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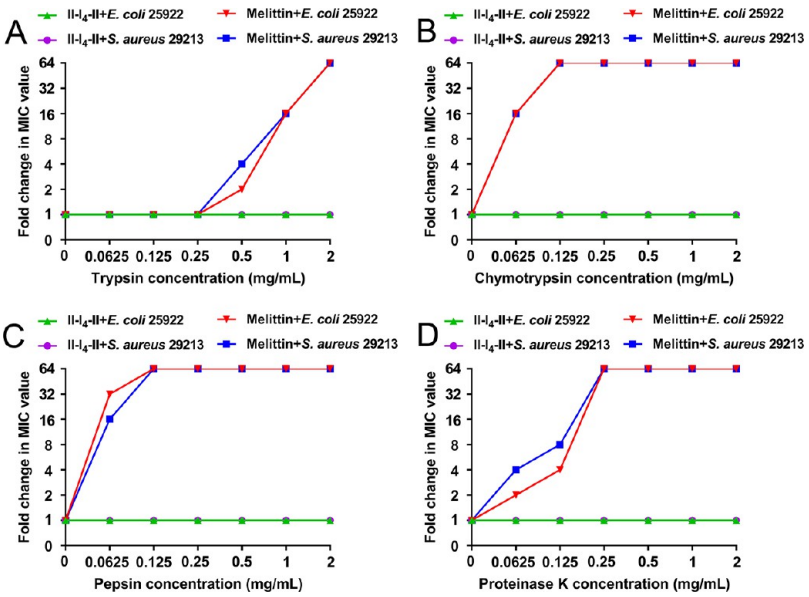

Figure 4. Fold change in the MIC value of the peptides against *E. coli* 25922 and *S. aureus* 29213 in the presence of proteases. The test was carried out three times independently with analogous results.

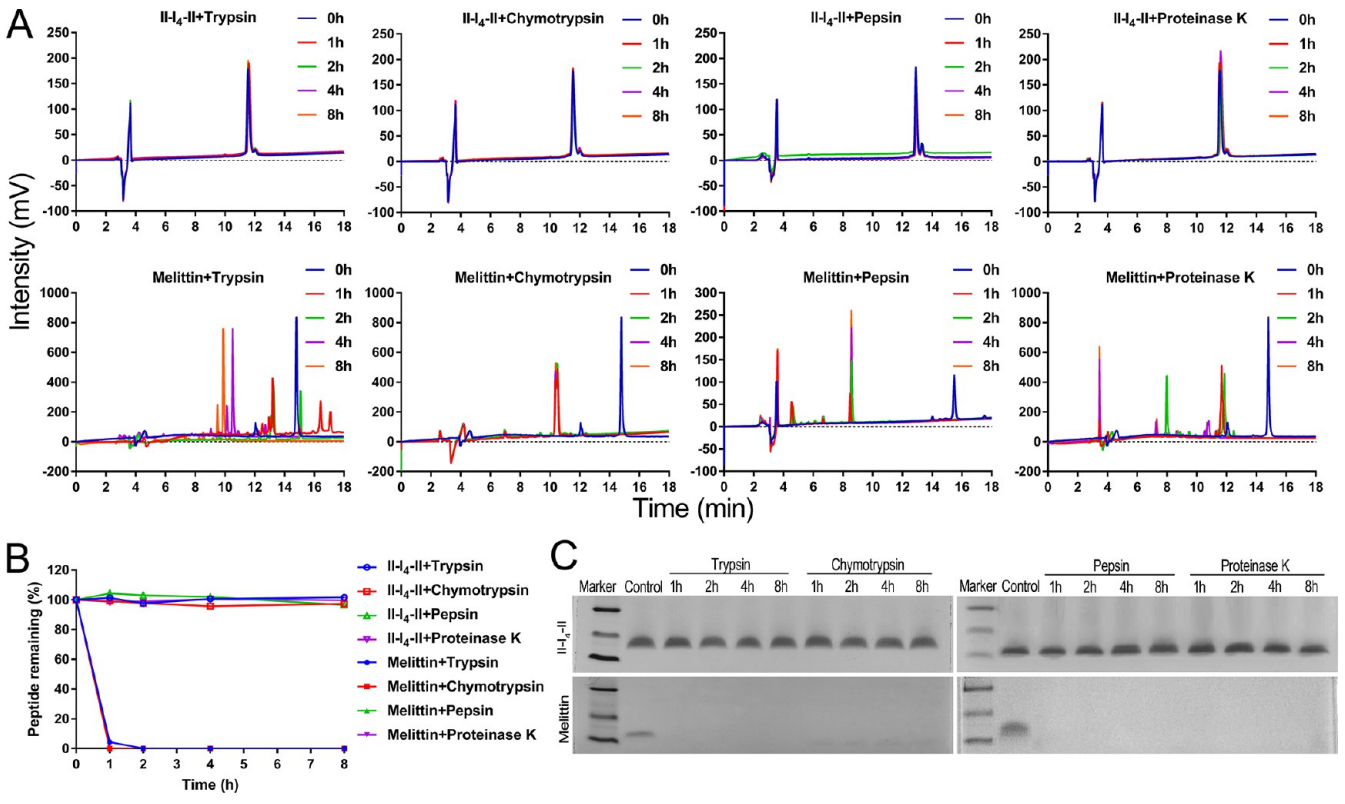

Figure 5. Effect of different duration on protease stability of the peptides. (A) RP-HPLC chromatographic peak of II-I_4-II and melittin showing degradation of the intact peptides at various time points after incubation with 2 mg/mL each of trypsin, chymotrypsin, pepsin (pH = 2.0), and proteinase K at 37 °C. (B) Retention rate of peptide in the presence of various proteases, which were calculated by integration of the peaks from the RP-HPLC profile. (C) Cleavage of II-I_4-II and melittin by proteases. The peptides were incubated with 2 mg/mL each of trypsin, chymotrypsin, pepsin (pH = 2.0), and proteinase K for 1, 2, 4, and 8 h at 37 °C. The MW of the marker from top to bottom corresponds to 14.4, 6.5, and 3.3 kDa.

the bacterial membrane, and thus leading to high salt stability, which was consistent with previous reports.[30,32,45]

Complex serum components were considered to be the main cause for the decrease of antimicrobial activity of AMPs.[46,47] The serum stability results (Table 4) showed that different concentrations of serum (25, 50, and 100%) slightly reduced the antimicrobial activity of all peptides, and high concentrations (100%) of serum had the greatest effect on the antimicrobial activity of these peptides, which may be associated with the concentration of anionic serum proteins (*e.g.*, serum albumin and lipoproteins) that could bind to AMPs and reduce their

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J. Med. Chem. 2020, 63, 9421−9435

effectiveness.[46,48,49] Overall, the end-tagged I-I_4-I and II-I_4-II peptides showed better serum stability than I_4.

Furthermore, to assess heat stability, the peptides were heated at 100 °C for 30, 60, and 90 min, during which the antimicrobial activity was slightly affected (Table S1). The heat stability of the designed peptides was comparable to that of melittin. For acid and alkali stability, the results revealed that the antimicrobial activity of the designed peptides increased slightly at different extreme pH values but with no significant regularity (Table S1).

Protease Stability. In the physiological salts, serum, acid, alkali, and heat stability tests, II-I_4-II showed high stability, and thus, II-I_4-II was selected for protease stability studies. High sensitivity to protease degradation is an important reason why most AMPs have not been successfully used clinically.[50] The MIC method was used to assess the impact of different protease concentrations on the antimicrobial activity of peptides. As shown in Figure 4, II-I_4-II maintained its original antimicrobial potency after treatment with 0.0625−2 mg/mL of proteases (trypsin, chymotrypsin, pepsin, and proteinase K). In contrast, the antimicrobial activity of melittin changed at low protease concentrations, and melittin was completely inactivated at high protease concentrations, which showed extremely low resistance to proteases. Subsequently, 16.5% tricine-SDS-PAGE and RP-HPLC were used to assess the impact of different exposure durations on the peptides. The RP-HPLC profile showed degradation of the peptides by proteases at various time points (Figure 5A,B). As shown in Figure 5A, within 8 h after each protease treatment, II-I_4-II exhibited a highly similar chromatographic peak compared with samples without protease treatment (0 h). Inversely, melittin was susceptible to the treatment with each protease, which resulted in an obvious change in the chromatographic peak at just 1 h. Figure 5B shows the percentage of remaining intact peptide. Consistent with the above results, II-I_4-II was nearly completely retained after each protease treatment within 8 h, while melittin was almost completely gone after each protease treatment for 1 h. The results of 16.5% tricine-SDS-PAGE further confirmed the protease resistance of the peptides (Figure 5C). II-I_4-II was resistant to each protease treatment and exhibited highly similar bands to the control. In contrast, the melittin bands indicated complete degradation by proteases within 1 h. The II-I_4-II peptide exhibited extremely high protease stability. These results indicate that the Cys and Lys at the N-terminal side and C-terminal side of Arg in the XX(XCRKPX)$_n$XX peptides completely block the action of trypsin on the C-terminal side of Arg, and that the Pro at the C-terminal side of Lys completely blocks the action of trypsin on the C-terminal side of Lys. Similarly, these results also suggest that Ile may not be the site specifically cleaved by chymotrypsin, pepsin, and protease K.[14,17,18] Conversely, the natural melittin sequence without proper arrangement contains abundant cationic and hydrophobic amino acids, which are excellent substrates for protease cleavage, and this may be the main reason for the inactivation of melittin. In conclusion, our design strategy effectively improved the protease stability of the peptide.

Resistance Development. Although the possibility of bacteria developing resistance to AMPs is thought to be low, resistance is still an important research parameter.[51] Thus, based on the analysis of the above studies, we assessed the resistance to II-I_4-II. The drug resistance of *E. coli* 25922 to II-I_4-II or ciprofloxacin during serial passaging at the sub-MIC concentration is shown in Figure 6. While ciprofloxacin resistance developed as early as passage 6 and the MIC value rise by 32-fold

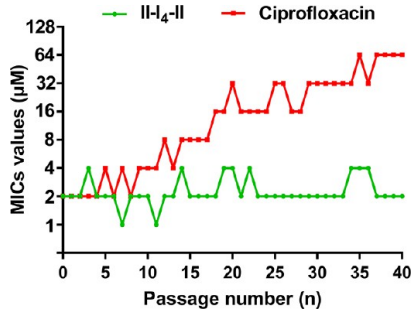

Figure 6. Resistance development of the peptide. Exposure of an II-I_4-II- and ciprofloxacin-sensitive strain of *E. coli* 25922 to sub-MIC dosing for 40 sequential passages. The test was carried out three times independently with analogous results.

after 40 passages, the antimicrobial potency of II-I_4-II was unaltered for the duration of the test. The above results show that the distinctive nonspecific membrane-disruptive mechanism of AMPs may provide a superiority in preventing resistance development.[15]

***In Vivo* Toxicity and Efficacy.** *In Vivo Toxicity.* As an overture for *in vivo* efficacy assay, we tested the potential toxicity of II-I_4-II in C57BL/6 mice through quantified scores, body weight change, and a series of functional parameters related to kidney and liver.[7] After systemic administration, mice in the control group (saline) and the low-dose group (10 mg/kg II-I_4-II) remained normally active, while a small number of mice in the high-dose group (20 mg/kg II-I_4-II) showed hunched and reduced activity 30 min before, and later returned to normalcy (Table S2). Moreover, there was no statistically significant change in body weight between the low-dose group and the high-dose group compared with the control group (Figure 7A). Blood urea nitrogen (BUN), creatinine (CREA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) levels in the low-dose group and the high-dose group, which are important indicators of kidney and liver injury, were not significantly different from those in the control group (Figure 7B,C).[7] The above results indicate that II-I_4-II at a dose of 10 mg/kg is unlikely to be toxic in mice.

In Vivo Efficacy. Compared with the highly effective antimicrobial activity of most AMPs *in vitro*, their performance *in vivo* is frustrating. Therefore, we evaluated the therapeutic potential of II-I_4-II *in vivo* through infection with *E. coli* 25922 in a peritonitis-sepsis model in C57BL/6 mice. Remarkably, treatment with II-I_4-II significantly reduced the bacteria load in multiple organs (Figure 8B); the corresponding median colony forming units (CFU) in the liver, spleen, kidney, and lung were 7.12, 6.02, 7.59, and 6.48 log CFU/g for the infection group and 5.08, 4.89, 5.38, and 5.58 log CFU/g for the treatment group (10 mg/kg of II-I_4-II), respectively, which showed an extremely significant difference. II-I_4-II effectively reduced the bacterial load in multiple organs (including the liver, spleen, kidney, and lung), suggesting that II-I_4-II has the potential to have antibacterial activity *in vivo*. Furthermore, histopathological examination revealed that II-I_4-II treatment significantly alleviated inflammation in multiple organs to different degrees compared with that in the infection group. Inflammatory cell infiltration in the lung and liver was reduced, and the bleeding in the kidney and spleen was reduced; these organs had no obvious lesions (Figure 8C). Moreover, we evaluated the levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in mouse serum.[52] As shown in Figure 8A,

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J. Med. Chem. 2020, 63, 9421−9435

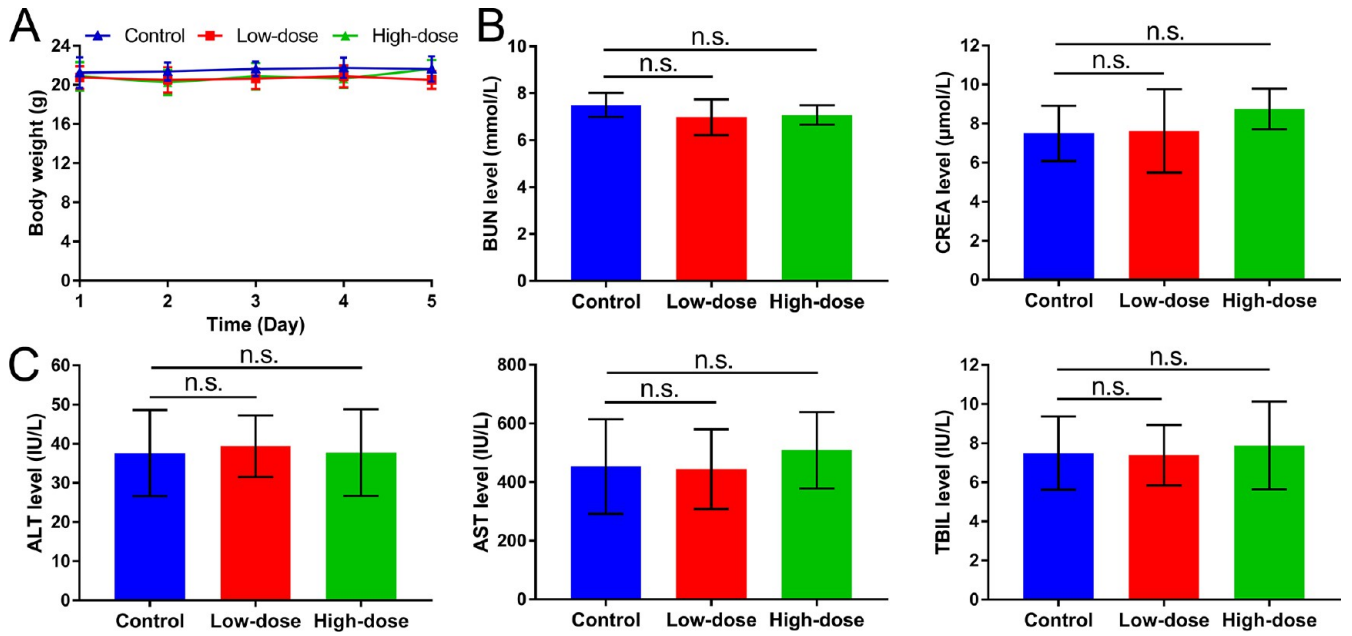

Figure 7. *In vivo* potential toxicity of II-I_4-II in C57BL/6 mice. (A) Body weight changes of C57BL/6 mice within 5 days upon administration of II-I_4-II (n = 8, female). (B) Levels of BUN and CREA in mice serum (n = 8, female). (C) Levels of ALT, AST, and TBIL in mice serum (n = 8, female). Data are the mean ± standard deviation. n.s. = no significance, Student's t test, significant difference from the control group.

treatment with II-I_4-II markedly reduced the levels of IL-1β, IL-6, and TNF-α compared with those in the infected group. IL-1β, which is considered to be a pro-inflammatory cytokine, is important for the host-defense response to acute infection.[53] IL-6 and TNF-α have been identified as primary mediators of sepsis and can cause septic shock through hemorrhage and severe pulmonary edema.[54,55] Therefore, the reduction of these pro-inflammatory cytokine indicates that II-I_4-II has a potential anti-inflammatory effect *in vivo*, which was consistent with our *in vitro* assays, indicating that II-I_4-II dose dependently inhibits the production of IL-1β, IL-6, and TNF-α in lipopolysaccharide (LPS)-induced mouse RAW 264.7 cells (Figure S3).

Antimicrobial Mechanism Research. Based on the above results, because II-I_4-II had the best *in vitro* and *in vivo* antimicrobial activity, high stability, and low toxicity, we used II-I_4-II to study the antimicrobial mechanism. Previous studies have proposed that most AMPs act through a membrane-disruptive mechanism.[13,51] To inspect the antimicrobial mechanism of II-I_4-II, the membrane permeation of Gram-negative bacteria and Gram-positive bacteria was studied using various fluorescent probes, and first-hand interactions between II-I_4-II and bacteria were observed under various microscopes.

LPS and Lipoteichoic Acid Binding. LPS and lipoteichoic acid (LTA) are the specific components with negative charge on the membrane surface of Gram-negative bacteria and Gram-positive bacteria, respectively, and they are also the main binding sites of most AMPs *via* electrostatic attraction.[56] As shown in Figure 9A, the LPS- and LTA-binding affinity of II-I_4-II and melittin increased in a dose-dependent manner, and the LPS- and LTA-binding affinity of II-I_4-II were comparable to that of melittin at the same concentration, illustrating that II-I_4-II can bind negatively charged components of the Gram-negative bacteria and Gram-positive bacteria membranes through electrostatic attraction, which is the first step in the interaction between peptides and bacteria.[57]

Outer Membrane Permeability. The outer membrane (OM) is a unique component of Gram-negative bacteria.[55] To investigate the OM permeability of the peptide, *E. coli* 25922 was selected as the model microorganism. *N*-phenyl-1-naphthylamine (NPN) is a fluorescent probe that fluoresces in a hydrophobic environment; when the peptide destroys the OM of the bacteria, NPN contacts the hydrophobic environment, causing an augment in fluorescence.[58] The results demonstrated that the membrane permeability of II-I_4-II and melittin performed in a dose-dependent manner, and the membrane permeability of both was comparable (Figure 9B), which indicates that II-I_4-II can permeabilize OM.

Cytoplasmic Membrane Depolarization. To explore the cytoplasmic membrane (CM) depolarization ability of the peptide, *E. coli* 25922 and *S. aureus* 29213 were selected as the model microorganism. Dipropylthiadicarbocyanine iodide [DiSC3(5)] is a cationic probe that gather in the CM under the influence of an internally negative membrane, causing self-quenching of fluorescence because of the polarized membrane surface and was released into the buffer upon disruption of the CM with a consequent augment in fluorescence.[8,59] As shown in Figure 9C, II-I_4-II disturbed the CM potential of *E. coli* 25922 and *S. aureus* 29213 at concentrations of 0.5, 1, and 2 × MIC, causing an increase in fluorescence intensity, which may be the result of CM destruction induced by II-I_4-II, resulting in generation of pores and ion channels that cause CM depolarization.[33] Similarly, melittin showed a similar trend in disrupting CM potential.

Confocal Laser Scanning Microscopy Imaging. Propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labeled II-I_4-II was used to observe the site of peptide action using confocal laser scanning microscopy (CLSM) (Figure 10). PI cannot pass through the cell membrane of living cells but it can traverse impaired cell membrane and embed into double-stranded deoxyribonucleic acid, releasing red fluorescence.[8] II-I_4-II was conjugated with FITC, which emits green fluorescence. As anticipated, the bacterial membrane was clearly delineated by the green fluorescence of FITC-labeled II-I_4-II (Figure 10B,F), and the red fluorescence of PI was detected inside the bacterial

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J. Med. Chem. 2020, 63, 9421−9435

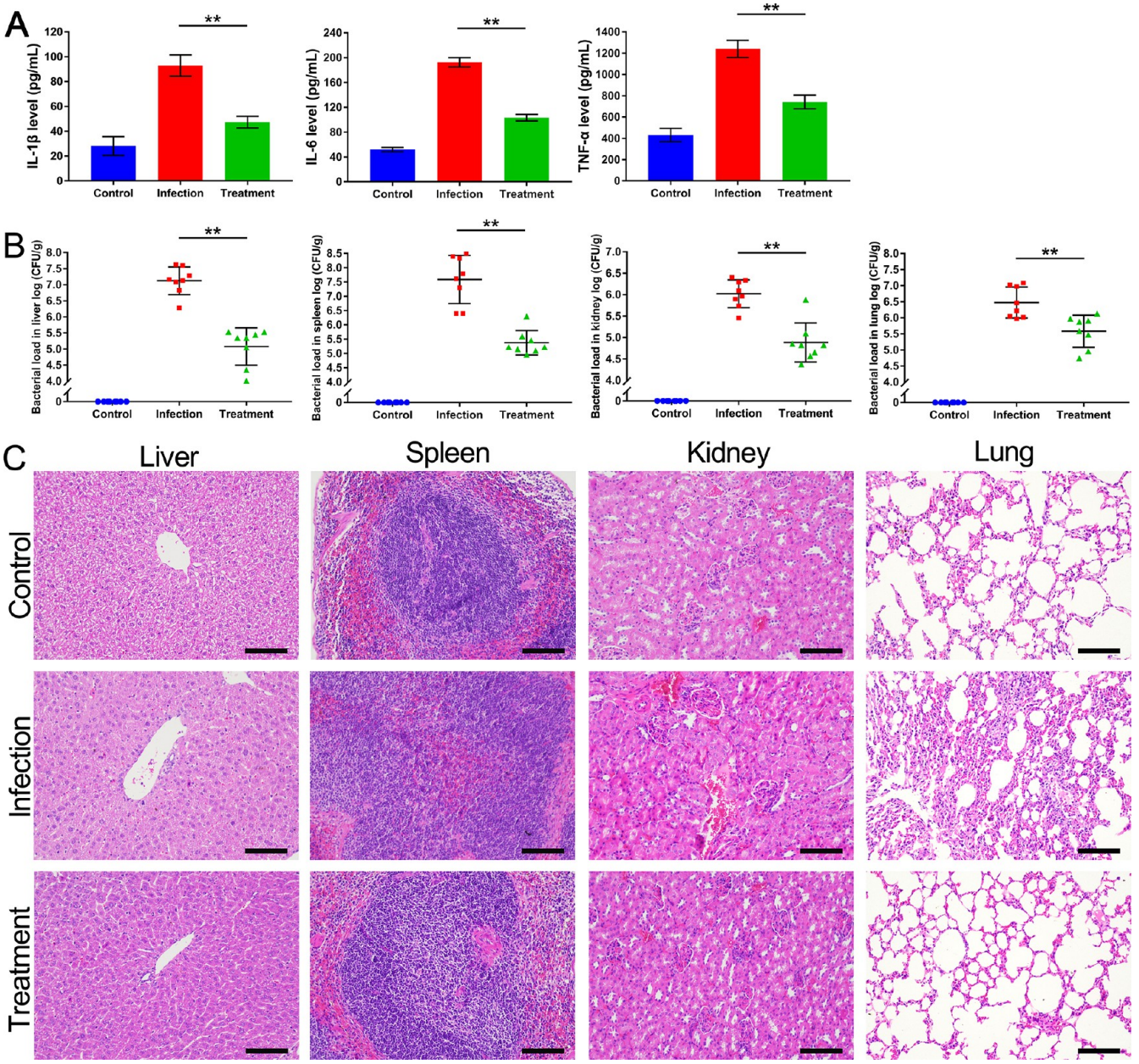

Figure 8. *In vivo* therapeutic potential of II-I_4-II in a peritonitis-sepsis C57BL/6 mouse model infected with *E. coli* 25922. (A) Levels of IL-1β, IL-6, and TNF-α in mice serum (n = 8, female). (B) Bacteria load in the liver, spleen, kidney, and lung (n = 8, female). (C) Images showing hematoxylin−eosin staining of the liver, spleen, kidney, and lung. The scale bar is 100 μm. Data are the mean ± standard deviation. **P < 0.01, Student's t test, significant difference from the infection group.

cell membrane (Figure 10C,G). Merged images show the localization of the peptide more intuitively (Figure 10D,H) and are consistent with the abovementioned membrane penetration results and other membrane-disruptive AMP cases.[60]

Membrane Integrity Analysis. Flow cytometry (FCM) and the nuclear fluorescent probe PI, which can traverse impaired cell membrane, were used to analyze the ability of the peptide to destroy bacterial membranes. Meanwhile, fluorescence microscopy SYTO 9 and PI were used to observe the ability of the peptide to destroy bacterial membranes.[61] As shown in Figure 11, the PI fluorescence signal in the control was at a very low level (*E. coli* 25922 and *S. aureus* 29213 were only 4.2 and 6.3%), indicating intact bacterial membranes. The percentage of membrane rupture (PI fluorescence signal) increased in a dose-dependent manner with II-I_4-II concentration. The II-I_4-II caused more than 90% of *E. coli* 25922 and *S. aureus* 29213 membranes to rupture at 2 × MIC. As anticipated, the fluorescence images showed a trend similar to that observed in the FCM results (Figure S4). These results further confirm that II-I_4-II kills bacteria by destroying the integrality of the bacterial membranes.

Scanning Electron Microscopy and Transmission Electron Microscopy Analyses. Scanning electron microscopy (SEM) was used to further visually observe the effect of the peptide on the membrane morphology of *E. coli* 25922 and *S. aureus* 29213 (Figure 12). In the absence of peptide treatment, the bacterial membranes of *E. coli* 25922 and *S. aureus* 29213 were smooth (Figure 12A,D). In contrast, the bacterial membranes of *E. coli* 25922 and *S. aureus* 29213 treated with II-I_4-II showed obvious corrugation, breakage, and fragmentation (Figure 12B,C,E,F).

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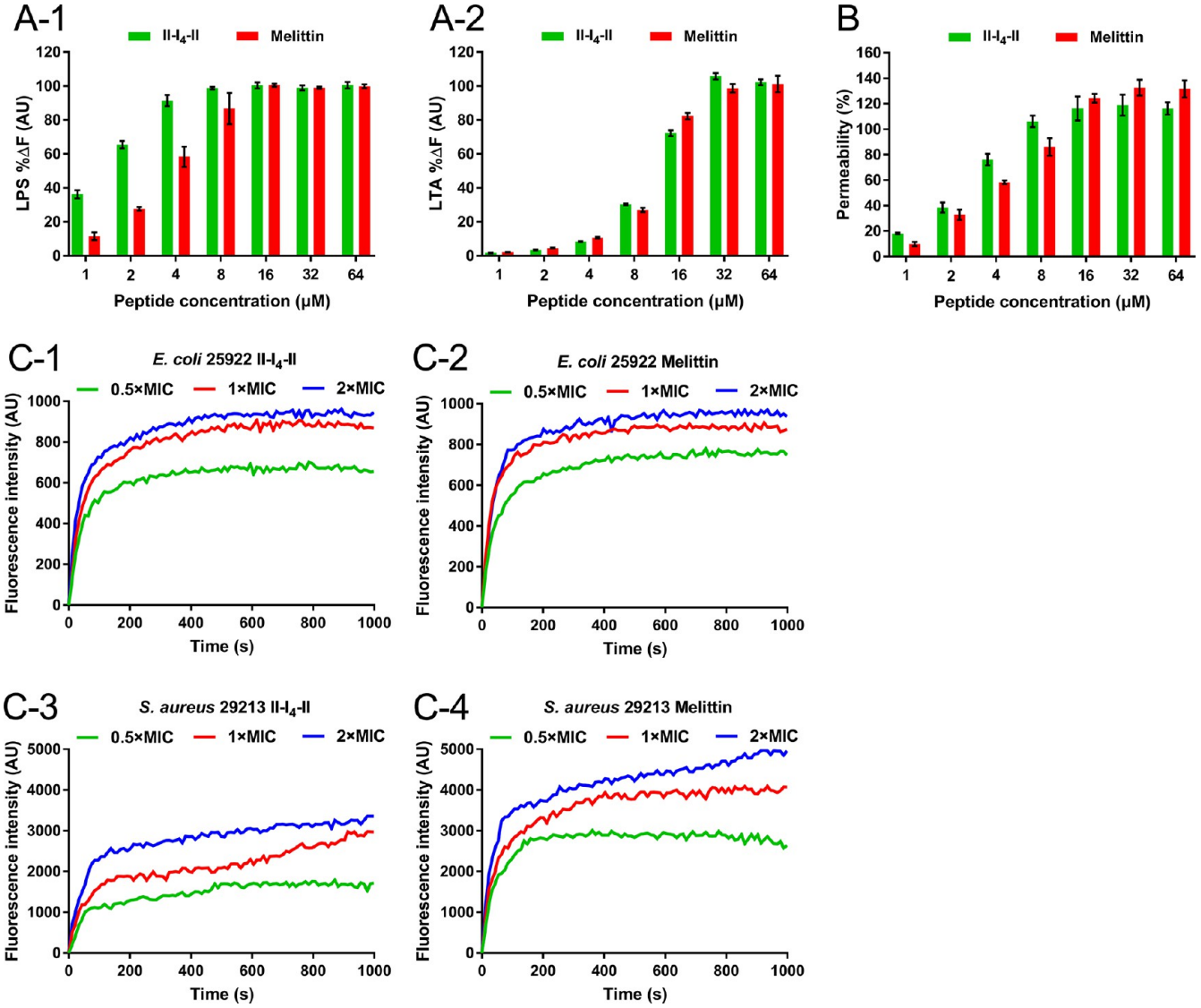

Figure 9. Preliminary antimicrobial mechanism of peptides. (A) LPS- and LTA-binding affinities of the peptides. (B) OM permeability of the peptides. (C) CM depolarization ability of the peptides.

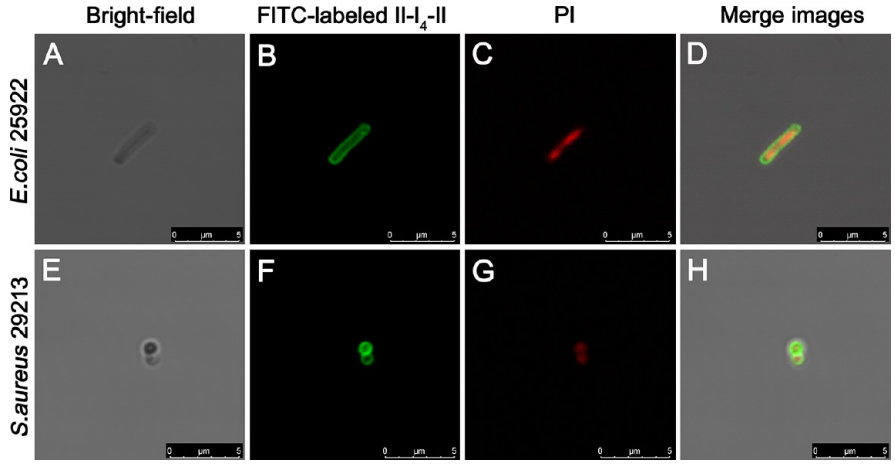

Figure 10. CLSM images of *E. coli* 25922 and *S. aureus* 29213. (A,E) Bright-field signal. (B,F) Green fluorescence signal of FITC-labeled II-I_4-II. (C,G) Red fluorescence signal of PI. (D,H) Merged images.

Transmission electron microscopy (TEM) was used to further visually observe the effect of the peptide on the ultrastructure of *E. coli* 25922 and *S. aureus* 29213 (Figure 13). Compared with the control bacterial cells, which showed a

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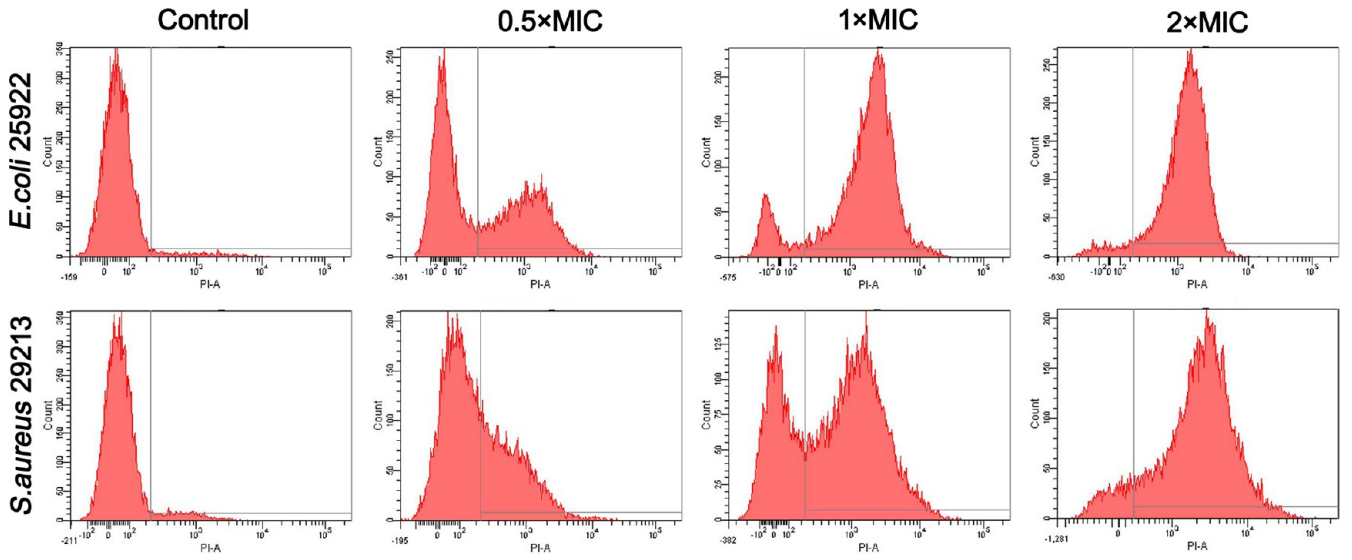

Figure 11. FCM analysis of the ability of peptide to destroy membranes of *E. coli* 25922 and *S. aureus* 29213.

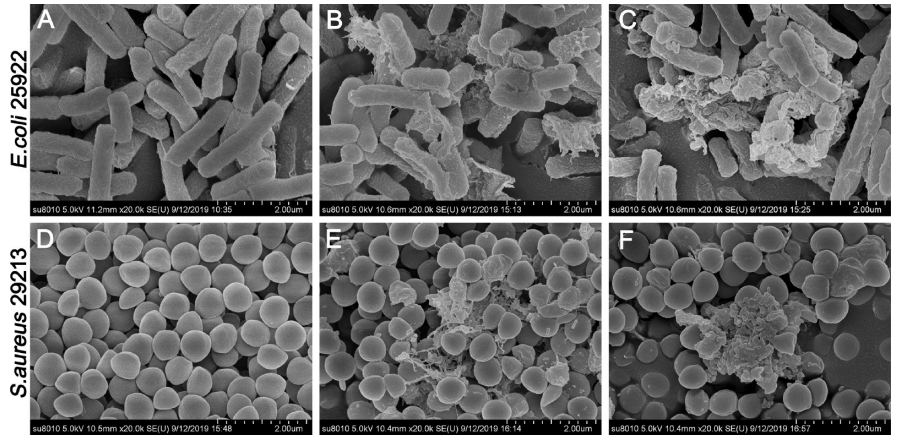

Figure 12. SEM micrographs of *E. coli* 25922 and *S. aureus* 29213. (A,D) Control. (B,C,E,F) II-I_4-II treated.

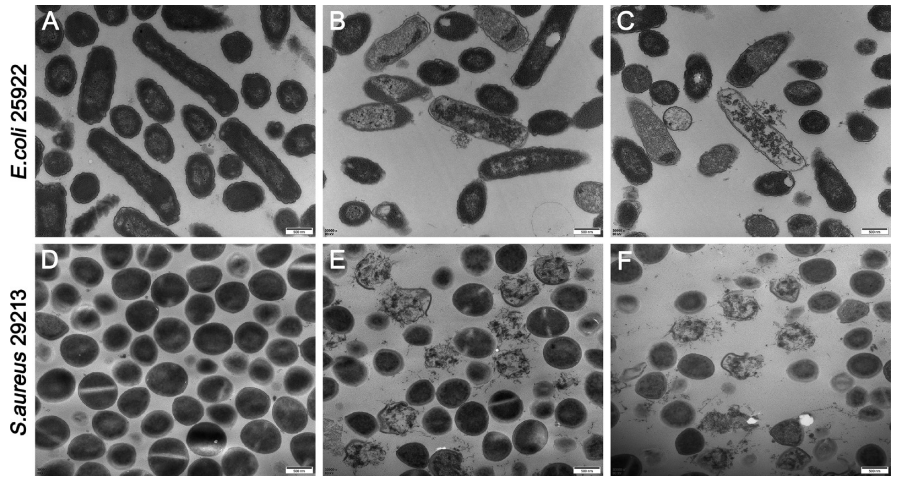

Figure 13. TEM micrographs of *E. coli* 25922 and *S. aureus* 29213. (A,D) Control. (B,C,E,F) II-I_4-II treated.

dense internal structure (Figure 13A,D), *E. coli* 25922 treated with II-I_4-II showed significant CM and OM separation, membrane rupture, cytoplasmic leakage, and sparsity (Figure 13B,C). Similarly, treated *S. aureus* 29213 exhibited severe membrane damage, cytoplasmic leakage, and sparsity (Figure 13E,F).

SEM and TEM images further verified that II-I_4-II caused bacterial death by destroying the bacterial membranes.

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J. Med. Chem. 2020, 63, 9421−9435

In summary, based on the above results we speculate that II-I_4-II first binds to the negatively charged components of the membrane surface through electrostatic attraction. Subsequently, II-I_4-II aggregates on the membrane surface, and when the threshold concentration is attained, its hydrophobic core is inserted into the phospholipid bilayer, leading to rupture of the bacterial membranes, divulgence of intracellular contents, and eventually death of the bacteria.

CONCLUSIONS

In the current paper, we presented an effective approach for the design of AMPs with high stability based on protease-specific cleavage sites and symmetrical end-tagging. By synthesizing a sequence of peptides and measuring their antimicrobial activity and biocompatibility, we found that the sequence length and the types of hydrophobic amino acids have notable effects on the antibacterial activity of peptides. Among these peptides, II-I_4-II possessed the best antibacterial activity (GM_{All} = 3.17) and the highest TI (TI_{All} = 40.32). More importantly, II-I_4-II exhibited extremely high stability in the presence of various proteases, physiological salts, and serum and under acid, alkali, and heat conditions; exhibited excellent therapeutic potential *in vivo*; and had strong anti-inflammatory ability *in vitro* and *in vivo*. The target peptide II-I_4-II aggregate on the membrane surface of Gram-negative bacteria and Gram-positive bacteria through electrostatic attraction, damaging the integrality of the bacterial membranes and causing divulgence of intracellular contents and eventually death of the bacteria, which may offer an advantage in preventing resistance development. In conclusion, our study confirms a potential method for designing AMPs with high stability, which might contribute to clinical translation of AMPs. Meanwhile, II-I_4-II has potential for development as a novel antibiotic.

EXPERIMENTAL SECTION

Bacterial Strains. The bacteria strains *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *S. typhimurium* C7731, *Salmonella pullorum* C7913, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, and methicillin-resistant *S. aureus* (MRSA) 43300 were obtained from the College of Veterinary Medicine, Northeast Agricultural University (Harbin, China). *Salmonella choleraesuis* CVCC 503 was kindly provided by the Chinese Academy of Agricultural Science (Beijing, China). *E. coli* UB1005 was kindly provided by the State Key Laboratory of Microbial Technology, Shandong University (Jinan, China). Clinical isolates of *S. aureus* 11011 were kindly obtained from the First Affiliated Hospital of Dalian Medical University (Dalian, China). *Streptococcus agalactiae* H92 was kindly obtained from the College of Animal Science and Technology, Guangxi University (Nanning, China).

Chemical Synthesis and Analysis of Peptides. All the peptides designed in this research were synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China) using solid phase peptide synthesis, and the MW of the peptides was confirmed by MS (Linear Scientific Inc, USA). Peptide purity was measured using RP-HPLC with a Gemini-NX C18-5 column (4.6 × 250 mm, 5 μm particle size). The peptide samples (20 μL) were analyzed in a 0.1% trifluoroacetic mobile phase component in water/acetonitrile at a flow rate of 1 mL/min and the wavelength of 220 nm for 30 min. The final purity of each peptide tested by RP-HPLC analysis was >95%.

CD Spectroscopy. The CD of the peptides (150 μM) was measured at wavelengths ranging from 195 to 250 nm using a J820 spectropolarimeter (Jasco, Tokyo, Japan) with a 1 mm path length cuvette in 10 mM PBS, 50% TFE, and 30 mM SDS. Finally, the mean residue ellipticity was calculated.

Determination of Antimicrobial Activity. The MIC of the peptides was determined using the standard microbroth dilution assay recommended by the Clinical and Laboratory Standards Institute (CLSI), with modifications.[62] After the bacteria were cultured in noncation-adjusted Mueller−Hinton broth (MHB, AoBoX) overnight, they were transferred to new MHB until reaching the exponential growth phase. Bacteria in the exponential phase were diluted to 2×10^5 CFU/mL with MHB. Briefly, 50 μL of the peptides and an equal volume of bacterial suspension were added to a 96-well plate with final concentrations of peptides ranging from 1 to 128 μM, after which the mixture was incubated for 18−24 h at 37 °C. Negative (MHB only) and positive (bacteria and MHB) controls were included. The optical density was tested with a microplate reader (Tecan GENios F129004, Tecan, Austria) at an absorbance wavelength of 492 nm. At least three independent assays were carried out.

Hemolytic Activity and Cytotoxicity Assays. The hemolytic activity of the peptide was assessed by determining the hemolytic rate of the peptide to hRBCs (volunteer donor: Yongjie Zhu, Harbin, China), and the cytotoxicity of the peptides was evaluated using the RAW 264.7 and HEK 293T, and the cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assays, as previously described.[41]

Salts, Serum, Acid, Alkali, and Heat Stability Assays. The salts, serum, acid, alkali, and heat stability of the peptides were assessed using the modified MIC method.[51] For the salts stability assay, each salt powder was dissolved in bovine serum albumin (Sigma-Aldrich) solution, and the peptides were incubated with physiological salts ions at different final concentrations (150 mM Na^+, 4.5 mM K^+, 1mM Mg^{2+}, 8 μM Zn^{2+},6 μM NH_4^+, and 4 μM Fe^{3+}). For the serum stability assay, the peptides were incubated in different concentrations (25, 50, and 100%) of human serum for 18−24 h at 37 °C. For the heat stability assay, the peptides were heated at 100 °C for different durations (30, 60, and 90 min) and then cooled to room temperature. For the acid and alkali stability assays, the peptides were incubated in PBS with different pH values (pH = 2, 4, 10, and 12) for 2 h. *E. coli* 25922 or *S. aureus* 29213 was used in the above assays, and the subsequent method was corresponded to the MIC test method. At least three independent assays were carried out.

Protease Stability Tests. The protease stability of the peptides was evaluated using an improved MIC method, 16.5% tricine-SDS-PAGE, and RP-HPLC.[39] To determine the impact of the protease concentration on the antimicrobial activities of the peptides, the peptides were incubated with different concentrations (0.0625, 0.125, 0.25, 0.5, 1, and 2 mg/mL) of different proteases [trypsin, chymotrypsin, pepsin (pH = 2.0), and proteinase K, Sigma-Aldrich] for 1 h at 37 °C. *E. coli* 25922 or *S. aureus* 29213 were used in the assays, and the subsequent method was corresponded to the MIC test method. At least three independent assays were carried out. To test the effects of protease incubation duration on peptides, the peptides were incubated with 2 mg/mL trypsin, chymotrypsin, pepsin (pH = 2.0), or proteinase K for 1, 2, 4, and 8 h at 37 °C. Subsequently, the above reaction solution was detected *via* 16.5% tricine-SDS-PAGE and RP-HPLC.

Drug Resistance Assays. The drug resistance of bacterial to the peptides was evaluated by the sequential passaging method. Ciprofloxacin was employed as the model antibiotic and *E. coli* 25922 as the model microorganism for the drug resistance assay. First, the MIC of the peptide and ciprofloxacin was determined, as described previously. Next, each sub-MIC concentration of bacteria was rediluted to 2×10^5 CFU/mL with MHB and was used for the next passage MIC determination. The above procedure was repeated for 40 passages.

***In Vivo* Toxicity and Efficacy Assays.** The protocols used in this test were approved by the Northeast Agricultural University Institutional Animal Care and Use Committee (NEAU-[2011]-9), and all the animal care and treatment regimens complied with the standards described in the "Laboratory Animal Management Regulations" (revised 2016) of Heilongjiang Province, China.

In Vivo Toxicity Assay. Twenty-four C57BL/6 female mice (Liaoning Changsheng Biotechnology Co., Ltd., Benxi, China) aged 6−7 weeks and weighing 20−22 g were randomly divided into the following three groups (n = 8): (a) control group (saline), (b) low-dose

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J. Med. Chem. 2020, 63, 9421−9435

group (10 mg/kg II-I_4-II), and (c) high-dose group (20 mg/kg II-I_4-II). After 3 days of prefeeding, 100 μL of saline or peptide solutions was injected into the peritoneal cavity of mice (saline, 10, and 20 mg/kg II-I_4-II from a to c group). The body weight and toxicity scores of mice were monitored continuously for 5 days. On the fifth day after administration, the mice were euthanized. Blood samples were obtained from the mice for measurement of serum biochemical levels.

In Vivo Efficacy Assay (Peritonitis-Sepsis Model). Twenty-four C57BL/6 female mice (Liaoning Changsheng Biotechnology Co., Ltd., Benxi, China) aged 6−7 weeks and weighing 20−22 g were randomly divided into the following three groups (n = 8): (a) control group (saline), (b) infection group (*E. coli*), and (c) treatment group (*E. coli* + II-I_4-II). After 3 days of prefeeding, 100 μL of saline was injected into the peritoneal cavity of mice in the control group, while other mice received 100 μL of *E. coli* 25922 (~1 × 10^8 CFU/mL) to initiate infection. The treatment group was treated with II-I_4-II (10 mg/kg) *via* intraperitoneal injection 1 h after infection, while the control group and infection group received saline. Then, 12 h after the infection, the mice were euthanized. At the same time, the liver, spleen, kidney, and lung of mice were taken out and weighed to analyze the bacterial load in those organs. For histopathological examination, liver, spleen, kidney, and lung sections were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. The level of IL-1β, IL-6, and TNF-α in the serum was analyzed with ELISA kits (Shanghai Jinma).

Anti-inflammatory Activity Tests. The anti-inflammatory activity of the peptide was evaluated by determining the concentrations of IL-1β, IL-6, and TNF-α produced by RAW 264.7 cells. In brief, RAW 264.7 cells (2 × 10^5 cells/well) were incubated with different concentrations of peptide and LPS (from *E. coli* O111:B4, 100 ng/mL) (24 h, 37 °C). The negative control was untreated RAW 264.7 cells, and the positive control was RAW 264.7 cells treated with LPS alone. The level of IL-1β, IL-6, and TNF-α in the cell supernatant was analyzed using ELISA kits (Shanghai Jinma).

Antimicrobial Mechanism Research. *LPS- and LTA-Binding Assays.* The LPS (from *E. coli* O111:B4, Sigma-Aldrich) and LTA (from *S. aureus*, Sigma-Aldrich) binding affinity of the peptides were assessed with a fluorescent probe BODIPY-TR-cadaverine (Thermo Fisher) displacement method, as previously described by Tan *et al.*[41]

OM Permeability Tests. As previously described by Yang *et al.*,[40] the OM permeability of the peptides was assessed using a fluorescent probe NPN (Sigma-Aldrich) method.

CM Depolarization Assays. The CM depolarizing ability of peptides against *E. coli* 25922 or *S. aureus* 29213 was detected by fluorescent probe DiSC3(5) (Thermo Fisher), as reported previously.[63] The fluorescence intensity of 0−1000 s was recorded.

CLSM Imaging. The action sites of the peptide were observed with PI (Thermo Fisher) and FITC *via* CLSM. *E. coli* 25922 or *S. aureus* 29213 in the exponential phase was centrifuged (5000*g*, 5 min, 4 °C) to discard the MHB, and the remaining bacteria were washed three times with PBS (10 mM, pH = 7.4) and resuspended with PBS to OD_{600nm} = 0.2. FITC-labeled II-I_4-II (1 × MIC) was incubated with the bacteria for 1 h at 37 °C. Next, the mixture was incubated with PI (10 μg/mL) for another 30 min at 4 °C. The excess FITC-labeled peptide and PI were washed with PBS, and then the 10 μL sample was transferred to a glass slide. Afterward, images were obtained using confocal microscopy (Leica TCS SP8, Germany).

FCM Analysis. The bacteria sample preparation steps were the same as those for CLSM. The peptide (0.5, 1 and 2 × MIC) was incubated with the *E. coli* 25922 and *S. aureus* 29213 for 1 h at 37 °C. Next, the mixture was incubated with PI (10 μg/mL) for another 30 min at 4 °C. A control without peptide treatment was also prepared. Then, the data were measured using FCM (Becton-Dickinson, USA) at an excitation wavelength of 480 nm.

Fluorescence Imaging. The integrity of bacterial membranes was observed with fluorescence microscopy (Life Technologies EVOS FL Auto, USA) by SYTO 9 (Thermo Fisher) and PI. The bacteria sample preparation steps were the same as those for CLSM. The peptide (0.5, 1 and 2 × MIC) was incubated with the bacteria for 1 h at 37 °C. Then, the mixture was incubated with SYTO 9 and PI for 30 min at 4 °C. Bacteria not treated with peptides served as a control.

SEM and TEM Analyses. Morphological and ultrastructural alterations of bacterial membranes were observed *via* SEM (Hitachi su-8010, Japan) and TEM (Hitachi H-7650, Japan), respectively. *E. coli* 25922 or *S. aureus* 29213 in the exponential phase was centrifuged (5000*g*, 5 min, 4 °C) to discard the MHB, and the remaining bacteria were washed three times with PBS (10 mM, pH = 7.4) and resuspended with PBS to OD_{600nm} = 0.2. The bacteria were incubated with the peptides (1 × MIC) for 1 h at 37 °C. The subsequent steps were the same as those described in our previous reports.[41]

Statistical Analysis. Quantitative data are represented as the mean ± standard deviation. For statistical analyses, analysis of variance was conducted using Statistical Product and Service Solutions 20.0 software, and comparisons between groups were conducted using Student's t test. Statistically, $P < 0.05$ was deemed to manifest a significant difference.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00583.

Acid, alkali, and heat stability of peptides; score of potential toxicity of II-I_4-II; RP-HPLC spectra of the peptides; CD spectra of the peptides; anti-inflammatory activity of II-I_4-II *in vitro*; and fluorescence microscopy images of *E. coli* 25922 and *S. aureus* 29213 (PDF)

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Author Contributions

Y.Z. and C.S. contributed equally to this work and are both co-first authors. Y.Z. and A.S. designed and conceived the

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J. Med. Chem. 2020, 63, 9421−9435

experiments. All experiments were completed by Y.Z., C.S., G.L., Z.L., P.T., Q.J., and B.C. The main manuscript text was written by Y.Z. This work was supervised by A.S. All of 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

AMPs, antimicrobial peptides; TFE, 2,2,2-trifluoroethanol; RP-HPLC, reversed-phase high-performance liquid chromatography; GM, geometric mean; CFU, colony forming units; OM, outer membrane; hRBCs, human red blood cells; H, mean hydrophobicity; MHC, minimum hemolysis concentration; CM, cytoplasmic membrane; TI, therapeutic index; IL-1β, interleukin-1β; NPN, *N*-phenyl-1-naphthylamine; LTA, lipoteichoic acid; IL-6, interleukin-6; PI, propidium iodide; DiSC3(5), dipropylthiadicarbocyanine iodide; CLSM, confocal laser scanning microscopy; FITC, fluorescein isothiocyanate; AST, aspartate aminotransferase; MHB, Mueller−Hinton broth; BC, BODIPY-TR-cadaverine; FCM, flow cytometry; ALT, alanine aminotransferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; CLSI, clinical and laboratory standards institute; CREA, creatinine; TBIL, total bilirubin

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