Graphical Abstract

Triterpene sapogenin-polyarginine conjugates exhibit promising antibacterial activity against Gram-positive strains

Leave this area blank for abstract info.

Heiya Na ^a, Xiangpeng Li ^a, Cunbin Zou ^a, Chenhong Wang ^a, Chao Wang ^{a,*}, and Keliang Liu ^{a,*}

^a State Key Laboratory of Toxicology and Medical Countermeasures, Beijing Institute of Pharmacology & Toxicology, 27 Tai-Ping Road, Beijing 100850, China

Triterpene sapogenin-polyarginine conjugates exhibit promising antibacterial activity against Gram-positive strains

Heiya Na^a, Xiangpeng Li^a, Cunbin Zou^a, Chenhong Wang^a, Chao Wang^{a,*} and Keliang Liu^{a,*}

^a State Key Laboratory of Toxicology and Medical Countermeasures, Beijing Institute of Pharmacology & Toxicology, 27 Tai-Ping Road, Beijing 100850, China

ARTICLE INFO

Article history:

Received

Received in revised form

Accepted

Available online

Keywords:

Triterpene sapogenin

Polyarginine peptide

Antibacterial activity

Click chemistry

Synergistic effect

ABSTRACT

Triterpene sapogenins are a group of biologically active compounds with antibacterial activity. However, the limited solubility and poor bioavailability of triterpene sapogenins restrict their therapeutic application. Polyarginine peptides are small cationic peptides with high affinities for multiple negatively charged cell membranes and possess moderate antibacterial activities. In this study, we designed and synthesized a series of sapogenin-polyarginine conjugates in which the triterpene sapogenin moiety was covalently appended to the positively charged polyarginine via click chemistry. A clear synergistic effect was found, and the conjugates exhibited potent and selective antibacterial activity against Gram-positive strains. Among them, BAc-R3 was the most promising compound, which was also proven to be nontoxic toward mammalian cells as well as stable in plasma. The mechanism of BAc-R3 primarily involves an interaction with the bacterial membrane, similar to that of antimicrobial peptides (AMPs). This scaffold design opens an avenue for the further development of novel antibiotics comprised of the combination of a peptide and a natural product.

2016 Elsevier Ltd. All rights reserved.

1. Introduction

Triterpenes are a group of naturally occurring secondary metabolites that are biosynthesized in plants through squalene cyclization.^[1] These secondary metabolites act as natural chemical barriers to prevent various pathogen and herbivore infections.^[2] Several pentacyclic triterpenes, such as betulinic acid (BA), have been shown to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* *in vitro*.^[3–5] Ursolic acid (UA) and its isomer oleanolic acid (OA) also have been confirmed to display inhibitory activity against different bacterial strains.^[3, 6] However, the antibacterial activities of these pentacyclic triterpenes are in the low micromolar range, and their low bioavailability limits their potential therapeutic applications.^[7] Therefore, structural modifications are needed to improve the biological activities and bioavailability of pentacyclic triterpenes.

It is known that some membrane-interacting peptides, referred to as cell-penetrating peptides (CPPs), function as efficient vehicles to deliver bioactive compounds into multiple cell types.^[8, 9] CPPs are generally small cationic peptides that often have amphiphilic properties.^[10] Currently, many studies have focused on investigating the antimicrobial activities of CPPs because they share the structural features of antimicrobial peptides (AMPs).^[9, 11–13] Polyarginine peptides (R_n , $n > 7$), a type of widely used CPPs, are able to enter mammalian and certain microbial cell membranes and are proven to inhibit the growth of

the Gram-positive bacteria *Bacillus cereus*, *S. aureus*, and *Micrococcus luteus*, with a moderate potency.^[14–18] Compared with many AMPs, polyarginine peptides are composed only of arginine residues and do not contain a hydrophobic moiety. A high content of cationic charge is crucial for the initial interaction between AMPs and the bacterial membrane, while the hydrophobic moieties of AMPs drive peptide insertion into the bacterial membrane, resulting in membrane permeabilization.^[19, 20] It is possible that the weak antimicrobial activities of polyarginine peptides are due to the absence of a hydrophobic moiety, which potentially decreases the extent of membrane insertion of the polyarginine peptides.

Accordingly, we speculated that conjugation of hydrophobic pentacyclic triterpenes with cationic polyarginine peptides would yield hybrid molecules that show synergistic effects. In this hybrid molecule, the pentacyclic triterpene could form a hydrophobic core and produce an amphiphilic structure when it was covalently conjugated with a polyarginine peptide. In addition, recent studies have investigated in conjugation of a peptide with natural products to improve the interaction with membranes as well as the antibacterial activity.^[21–24] In an exemplary work, Shai et al. designed and synthesized a series of compounds in which different length of aliphatic acids were covalently attached to a cationic tetrapeptides.^[21] These compounds exhibited potent antimicrobial activity against a variety of microorganisms. Similarly, Chukung and coworkers

* Corresponding author. For K. L.: Tel: +86-10-6816-9363; fax: +86-10-6821-1656; e-mail: keliangliu55@126.com

* Corresponding author. For C. W.: Tel: +86-10-6693-0640; fax: +86-10-6821-1656; e-mail: chaow301@gmail.com

demonstrated that conjugation with fatty acid might promote the antimicrobial activity of peptide by enhancing their ability to form secondary structures upon interaction with bacterial membranes.^[22] Liu et al. made a short amphiphilic cholesterol-peptide conjugate that was composed of TAT, six arginine residues and cholesterol. This peptide formed core-shell structured nanoparticles with strong antimicrobial activity against a range of bacteria, fungi and yeast.^[23] Likewise, it has revealed that the incorporation of different amino acids, especially positively charged amino acids, to natural products might improve the water solubility and bioavailability as well as increase their biological activities.^[25, 26] In addition, polyarginine peptides, which are rich in cationic charges, have high affinities for bacterial membranes and play an important role as they selectively interact with the membrane.^[27] Thus, the fusion of polyarginine peptides with pentacyclic triterpenes would possibly change the physicochemical properties and enhance the antimicrobial activities of bioactive triterpenes and polyarginine peptides. In this study, a novel group of triterpene sapogenin-polyarginine conjugates were designed and synthesized. BA and UA acted as the hydrophobic moieties, and different lengths of polyarginine were appended to fine-tune the cationicity and solubility of the conjugates. These sapogenin-polyarginine conjugates were tested for their antibacterial activities against both Gram-positive and Gram-negative strains. Their cytotoxicity to mammalian cells (human erythrocytes, 3T3 cells, and MCF-7 cells), mechanism of action, and antibacterial activity in the presence of plasma were also evaluated.

2. Results and Discussion

2.1 Synthesis

In this study, all of the triterpene sapogenin-polyarginine conjugates were synthesized by the click chemistry reaction.^[28] To carry out this reaction, an alkynyl group was introduced to two triterpene sapogenins, and polyarginine was functionalized on the N-terminus with an azido moiety. Briefly, two series of triterpene sapogenin derivatives (XAc and XAo series) were prepared as reported previously.^[24] They were synthesized at the C₃ and C₂₈ positions, respectively (Figure 1). For the modification of polyarginine, an azido moiety was introduced at the N-terminus of polyarginine by acylation with azido acetic acid. The number of arginine residues varied from one, two, three, five, and eight, respectively; thus, we obtained five azido peptide precursors in this manner. Thereafter, the triterpene sapogenin derivatives were covalently attached to the azido peptide precursors under mild conditions via click chemistry. Accordingly, 20 triterpene sapogenin-polyarginine conjugates were synthesized in this study. They were purified by preparative reversed-phase HPLC, and their purity (> 95%) was determined by analytical HPLC. All of the compounds were characterized by LC-MS or HR-MS, and their theoretical molecular weights (MW_{obs.}) were identical to the calculated molecular weights (MW_{calc.}).

Figure 1. Chemical structures of the triterpene sapogenin derivatives.

2.2 Antibacterial activity

The antibacterial activities of all the conjugates were tested against both Gram-positive and Gram-negative strains, including *S. aureus* (ATCC 25923), *B. subtilis* (ATCC 6633), and *E. coli* (ATCC 25922). The activity was demonstrated by their minimum inhibitory concentrations (MICs), which are listed in Table 1. The MICs of these conjugates were 1.8–83.0 μM and displayed more potent antimicrobial activities than triterpenes (68–547 μM) and polyarginine (12.3 to > 2890 μM), suggesting that the sapogenin-polyarginine conjugates exhibit a clear synergistic effect. To clarify whether triterpenes and polyarginine in a mixture state also resulted in significant synergistic effect as in a covalently linked state, the antibacterial activity of a mixture of BA and R3 (1:1 ratio, unlinked) was tested as the control. Although BAc-R3 exhibited promising antibacterial activity, the synergy was lost when simply mixing BA with R3 in 1:1 ratio, which displayed no potency even at 531 μM . Except for the XAc-R1 and XAo-R1 conjugates, the MIC values against Gram-positive bacteria (1.8–8.6 μM) were comparable to those of MSI-78 (1.5 μM , peptide sequence is GIGKFLKKAKKFGKAFVKILKK), which is a cationic amphiphilic AMP currently undergoing phase III clinical trials for diabetic foot ulcers.^[29, 30] Of note, the conjugates in which the triterpene was linked with the non-cell penetrating peptide R3 displayed the most promising antibacterial activities. These results indicated that the antibacterial activities of these triterpene sapogenin-polyarginine conjugates were not due to the contributions of the triterpene or polyarginine alone but to the combination of the hydrophobic and cationic moieties of the amphiphilic scaffold.

Interestingly, these conjugates showed greater susceptibility against Gram-positive bacteria than Gram-negative bacteria, implying that these conjugates exhibit antibacterial specificity to Gram-positive strains. All of the sapogenin-polyarginine conjugates were generally not sensitive or less sensitive to Gram-negative bacteria. As observed in Table 1, all the XAc-polyarginine conjugates showed no inhibitory activities against *E. coli*, even at 66 μM . In contrast, the XAo-R3 conjugates were found to be moderately active against *E. coli*, with an MIC value of 14.7 μM . Overall, XAo-R3 series showed higher inhibitory activities against *E. coli* than that of XAc-R3 series, suggesting that the sapogenin position that is conjugated to R3 has a relatively important effect on the antibacterial activities. On the other hand, the MIC values were closely correlated with the number of arginine residues in the polyarginine. As for the XAo series, only the conjugates with three arginine residues exhibited moderate inhibitory activity (MIC = 14.7 μM). Fewer or more arginine residues eliminated the activity of the conjugates, as shown in Table 1. Additionally, the water solubility of the BAO-polyarginine conjugates was tested to assess the correlation between the solubility and antibacterial activities of conjugates (Table S1). As shown in Table S1, polyarginine length-dependent increased water solubility was found in BAO-polyarginine conjugates. It was noted that the solubility BAO-polyarginine conjugates showed no positive correlation with the antibacterial activities toward all three bacteria combined with antibacterial data.

2.3 Hemolytic activity

Hemolysis is one of the major parameters to assess the ability of cationic peptides to disrupt the membrane integrity of mammalian cells.^[20] The hemolytic activity in this study was

represented by their HC₅₀ (i.e., the concentration at which 50% human erythrocytes are lysed) values and was determined with all designed sapogenin-polyarginine conjugates at concentrations ranging from 6.25 to 800 µg/mL. As illustrated in Table 1, the HC₅₀ values of these conjugates were 31 to > 800 µg/mL. The majority of the XAc series compounds that were linked to various numbers of arginine residues possessed no significant effect on human erythrocytes, even at a concentration of 800 µg/mL. However, compared with the XAc series conjugates, the

XAo series conjugates showed higher hemolysis. As for the same sapogenins, conjugates with polyarginine peptides at position C₃ showed significantly lower hemolytic activities (XAc series, HC₅₀ values ranging from 126 to > 800 µg/mL) than those conjugated at position C₂₈ (XAo series, HC₅₀ = 31–298 µg/mL). The results suggested that the substitution position of polyarginine on the sapogenin had a systemic effect on the hemolytic activities of these conjugates. Additionally, the length of polyarginine exhibited little effect on the HC₅₀ value.

Table 1
The antibacterial activities (MICs) of triterpene sapogenin-polyarginine conjugates

Compound	MIC (µg/mL and µM) ^[a]			HC ₅₀ (µg/mL and µM)	TI	
	<i>S. aureus</i> (ATCC 25923)	<i>B. subtilis</i> (ATCC 6633)	<i>E. coli</i> (ATCC 25922)		<i>S. aureus</i>	<i>B. subtilis</i>
BA	250 (547)	250 (547)	>500 (>1095)	ND ^[b]	ND	ND
UA	62.5 (137)	31.2 (68)	>500 (>1095)	ND	ND	ND
R1	>500 (>2890)	>500 (>2890)	>500 (>2890)	ND	ND	ND
R2	>500 (>1520)	>500 (>1520)	>500 (>1520)	ND	ND	ND
R3	>500 (>1031)	>500 (>1031)	>500 (>1301)	ND	ND	ND
R5	>500 (>627)	250 (313)	>500 (>627)	ND	ND	ND
R8	31.2 (24.6)	15.6 (12.3)	250 (197)	ND	ND	ND
BAC-R1	>125 (>157)	15.6 (19.6)	>125 (>157)	>800 (>1006)	ND	>51
UAc-R1	62.5 (78.6)	15.6 (19.6)	>125 (>157)	126 (158)	2	8
BAo-R1	>125 (>166)	62.5 (83)	>125 (>166)	298 (397)	ND	5
UAo-R1	7.8 (10.4)	3.9 (5.2)	>125 (>166)	62 (83)	8	16
BAC-R2	7.8 (8.2)	3.9 (4.1)	>125 (>131)	>800 (>841)	>103	>205
UAc-R2	7.8 (8.2)	3.9 (4.1)	>125 (>131)	ND	ND	ND
BAo-R2	7.8 (8.6)	3.9 (4.3)	>125 (>138)	ND	ND	ND
UAo-R2	3.9 (4.3)	3.9 (4.3)	>125 (>138)	44 (49)	11	11
BAC-R3	3.9 (3.5)	1.9 (1.8)	>125 (>113)	238 (215)	62	125
UAc-R3	7.8 (7.0)	3.9 (3.5)	>125 (>113)	399 (360)	51	102
BAo-R3	3.9 (3.7)	3.9 (3.7)	15.6 (14.7)	85 (80)	22	22
UAo-R3	3.9 (3.7)	3.9 (3.7)	15.6 (14.7)	31 (29)	8	8
BAC-R5	3.9 (2.7)	3.9 (2.7)	>125 (>88)	458 (323)	117	117
UAc-R5	7.8 (5.5)	3.9 (2.7)	>125 (>88)	ND	ND	ND
BAo-R5	7.8 (5.7)	3.9 (2.8)	>125 (>91)	48 (35)	6	12
UAo-R5	7.8 (5.7)	3.9 (2.8)	>125 (>91)	43 (25)	6	11
BAC-R8	7.8 (4.1)	7.8 (4.1)	>125 (>66)	>800 (>424)	>103	>103
UAc-R8	7.8 (4.1)	7.8 (4.1)	>125 (>66)	>800 (>424)	>103	>103
BAo-R8	7.8 (4.2)	7.8 (4.2)	>125 (>68)	102 (55)	13	13
UAo-R8	7.8 (4.2)	7.8 (4.2)	>125 (>68)	75 (41)	10	10
BA+R3	>500 (>531)	>500 (>531)	>500 (>531)	ND	ND	ND
MSI-78	3.9 (1.5)	3.9 (1.5)	7.8 (3.1)	385 (153)	100	100

^a MICs were determined from the mean value of three independent experiments repeated in triplicate

^b Not determined

To evaluate the selectivity of compounds toward bacterial cells over mammalian cells, the therapeutic index (TI) was calculated as follows: TI = HC₅₀ / MIC (Table 1). The TIs of all the conjugates ranged from 2 to > 205, indicating a selectivity of these conjugates toward bacterial cells, compared with that of mammalian cells. Altogether, the TI values of seven conjugates

were more than 50 against both Gram-positive bacteria studied. Furthermore, six conjugates showed TI values of more than 100 against *B. subtilis*, including the most potent compound BAC-R3. On the other hand, it was concluded that the TI values of the XAc series conjugates were generally higher than those of the XAo series. The XAc and XAo series conjugates with the number of

arginine residues ranging from 2 to 8 possessed comparable MIC values (1.8–8.6 μM), while their TI values varied widely (between 6 and > 205) due to differences of HC_{50} values. Overall, these results revealed that the conjugates were selectively toxic toward bacterial cells.

2.4 Cytotoxicity

Cytotoxic assays were performed to further analyze the selectivity of the conjugates toward bacteria over mammalian cells. The conjugate BAc-R3 was tested on the mammalian cell lines 3T3 and MCF-7 by the MTS assay (Figure 2). No toxicity was observed for BAc-R3 against MCF-7 cells at a concentration of 62.5 $\mu\text{g/mL}$, which is 20-fold of its MIC against *B. subtilis*. Additionally, even at 1000 $\mu\text{g/mL}$ BAc-R3, the cell viability of 3T3 cells was still more than 50%. These results indicated that BAc-R3 exhibits a good selectivity toward bacterial cells over mammalian cells (3T3 cells, MCF-7 cells, and human erythrocytes).

Figure 2. Cell viability of MCF-7 and 3T3 cells after treatment of different concentrations of BAc-R3.

2.5 Susceptibility to plasma

Poor protease stability severely limits the clinical use of many therapeutic peptides. Susceptibility to proteases is considered to be one of the drawbacks to many AMPs.^[31] Herein, we assessed the stability of the best compound BAc-R3 by using HPLC profile of the compound after incubation with blood plasma at different time points. MSI-78 was used as control. Briefly, BAc-R3 and MSI-78 were incubated with blood plasma, respectively. Eleven sample-plasma mixtures were collected sequentially over an 18 h period and centrifuged immediately. Then the residual compounds were quantified by HPLC. As shown in Figure 3 (a), the MSI-78 concentration decreased quickly after incubation for 6 h, remaining 54 % of the original amount, and only 1 % for 18 h. Compared with MSI-78, BAc-R3 remained 87 % of the original amount for 18 h, indicating that the BAc-R3 was much more stable in blood plasma than MSI-78. In addition, we further investigated the stability of BAc-R3 with a subsequent decrease in antibacterial activity in the presence of blood plasma as reported previously.^[32, 33] The results are shown in Figure 3 (b), which illustrates the MICs of the sample-plasma mixture at different time points against *S. aureus* (MIC = 3.5–7.0 μM). The sample-plasma mixture showed a two-fold decreased MIC value as that of the sample in the absence of plasma (MIC = 3.5 μM) after 3 h of incubation and maintained this MIC value until 18 h, which further confirmed that BAc-R3 exhibits a adequate stability in blood plasma *in vitro*.

Figure 3. In vitro stability of BAc-R3 in plasma. (a) Residual compounds of BAc-R3 and MSI-78 after incubation with blood plasma in different time points. (b) MIC values of sample-plasma mixtures at different time points against *S. aureus*. The results were determined from the mean value of three independent experiments repeated in triplicate.

2.6 Mechanism of action

Previous work has indicated that the cell membrane is the main target of many cationic small molecules and AMPs mainly due to the strong interactions of the cationic moieties of these compounds with the negatively charged bacterial cell membrane.^[30] With the cationic and amphiphilic structures of these conjugates, it was therefore hypothesized that they would also adopt a similar mode of action, causing membrane disruption to inhibit bacterial growth. As such, microscopic techniques were conducted to study the impact of the conjugates on the bacterial membrane.

2.6.1 Confocal laser scanning microscopy

To determine whether the mode of action of these conjugates involves bacterial membrane permeabilization, we used the dyes 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) and examined the staining by confocal laser scanning microscopy (Figure 4). DAPI is a double-stranded DNA-binding dye that stains all bacterial cells blue, regardless of their viabilities. PI is another type of dye that can pass through a damaged cell membrane and only stains dead cells red. After incubation of BAc-R3 with *S. aureus* for 2 h, large numbers of *S. aureus* cells showed both blue and red fluorescence as a result of DAPI and PI staining. In contrast, untreated *S. aureus* cells were only stained blue by DAPI, and few of them were stained by PI. These results clearly revealed that BAc-R3 was able to compromise the bacterial membrane.

2.6.2 Transmission electron microscopy (TEM)

To further understand the mechanism of antibacterial activity of these conjugates, TEM was used to evaluate individual morphological changes of *S. aureus* before and after treatment with $4 \times \text{MIC}$ of BAc-R3 for 2 h (Figure 5). As shown in Figure 5(a), the untreated bacteria remained as normal and intact spheres with a smooth surface. While in the treated group (Figure 5b), the cell structure was collapsed and the cellular contents were released. Obviously, the cell membrane of *S. aureus* was broken and cell lysis occurred. Therefore, this experiment confirmed that these conjugates primarily interacted with the bacterial cell membrane, similar to many natural cationic AMPs.

Figure 4. Confocal laser scanning microscopic images of *S. aureus* cells treated with $4 \times \text{MIC}$ of BAc-R3 for 2 h. **a1-a3**, images of control cells stained with PI, DAPI, or merged. **b1-b3**, images of BAc-R3-treated cells stained with PI, DAPI, or merged.

Figure 5. TEM images show the morphologies of *S. aureus* before (a) and after (b) incubation with $4 \times \text{MIC}$ of BAc-R3 for 2 h.

3. Conclusion

A group of cationic, amphiphilic, and novel triterpene sapogenin-polyarginine conjugates was developed. Encouragingly, these conjugates showed significant synergistic effects on the biological activity. As illustrated, the triterpene sapogenin-polyarginine conjugates displayed potent antimicrobial activities and high TI values against Gram-positive bacteria. Similar to AMPs, these conjugates were found to inhibit bacterial growth primarily via membrane disruption. In addition, they were selectively toxic toward bacterial cells over mammalian cells and resistant to degradation in blood plasma *in vitro*. With the encouraging results from these triterpene sapogenin-polyarginine conjugates and the urgent need to develop a new generation of antibiotics, this study provides useful insights for the rational design of novel antibiotics comprised of the combination of natural products and peptides.

4. Experimental Section

4.1 General synthetic information

In this study, all reagents purchased from commercial sources were used directly without further purification. For peptide synthesis reactions, the solvents were dried before use. While for other reactions, the solvents were used as received. Thin-layer chromatography was used to monitor reactions using silica gel GF254 plates from Qingdao Haiyang Chemical Company (Qingdao, China). BA and UA were purchased from Energy Chemical Company (Shanghai, China). Triterpene sapogenin derivatives were purified by column chromatography using silica gel (200–300 mesh) from Qingdao Haiyang Chemical Company (Qingdao, China). Fmoc-Arg(Pbf)-OH was purchased from GL

Biochem Ltd. (Shanghai, China). Rink amide resin was from Nankai Hecheng S&T Co. Ltd. (Tianjin, China). *N*, *N*'-dicyclohexylcarbodiimide (GL Biochem Ltd.) and *N*-hydroxybenzotriazole (GL Biochem Ltd) were used as coupling reagents. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra of compounds were recorded by a JNM-ECA-400 spectrometer (JEOL, Japan) in CDCl_3 . Mass spectra were measured on an API-150 mass spectrometer from ABI Inc. (USA).

4.2 Preparation of polyarginine

Polyarginine was synthesized by standard 9-fluorenylmethoxy carbonyl (Fmoc) chemistry with a solid-phase system.^[34] The Fmoc-protecting group was removed by 25% piperidine/*N*, *N*-dimethylformamide (DMF). The resin was washed three times (3 min each) with DMF, methanol, and dichloromethane, respectively, with the removal of the Fmoc-protecting group or coupling of a new amino acid each time. To obtain the azido peptide precursor, the N-terminus of the peptide was attached with azido acetic acid using HOBt/HBTU/DIEA as coupling reagents. After completion of the whole sequence, the synthesized peptides were cleaved from the resin with a mixture of trifluoroacetic acid/water/anisole/1, 2-ethanedithiol/*m*-cresol (82.5:5:5:2.5:5, v/v). The crude peptides were purified on a preparative reversed-phase high-performance liquid chromatography system (PrepLC 4000, SHIMADZU, Japan) with a Waters X-bridge C8, 10 μm , 19.5 mm $\times$ 250 mm column. Peptide purities were detected on an analytical RP-C8 column (Zorbax Eclipse XDB-C8, 5 μm , 4.6 mm $\times$ 150 mm) and confirmed to have > 98% purity. The molecular weight of the peptides was confirmed by LC-MS (ABI Inc., USA).

4.3 Bacterial strains

The bacterial strains used in this study included Gram-negative *E. coli* (ATCC 25922) and Gram-positive *S. aureus* (ATCC 25923) and *B. subtilis* (ATCC 6633). All strains were maintained in 80% sterile glycerol at -80°C . After being thawed, the aliquots were diluted (1:2500) in fresh sterile broth and grown overnight at 37°C with agitation.

4.4 Antibacterial assay

The MICs of compounds were determined by the broth microdilution method. Briefly, sterile 96-well plates (Corning, Inc., Corning, NY) were filled with 100 μL of sterile broth (pH 7.4) containing 3 g of Lab-Lemco Powder (Oxoid), 10 g of peptone (Oxoid), and 5 g of NaCl in deionized water to a final volume of 1 L. Then, portions (100 μL) of samples dissolved in saline at a concentration of 1000 $\mu\text{g/mL}$ were added to the first line of plates, each sample repeated in triplicate, and two-fold serial dilutions resulting in final concentrations ranging from 3.9 to 500 $\mu\text{g/mL}$ were prepared with sterile broth. Diluted (1:1000) bacterial suspensions (10 μL ; approximately 1.0×10^6 CFU/mL) were then added to the plates and incubated with two-fold dilutions of samples at 37°C for 16 h with continuous shaking. The MIC was determined by visual observation combined with absorbance measurement at 600 nm with a SpectraMax® M5 spectrophotometer (Molecular Devices, USA). The MIC was defined as the lowest concentration of compound required to completely inhibit bacterial growth. MSI-78 was used as a positive control, while cultures without samples were used as a negative control.

4.5 Hemolytic assay

The hemolytic activity of compounds was determined by measuring the amount of hemoglobin released by lysis of human erythrocytes. Fresh human red blood cells (hRBCs) obtained from heparinized human blood from the 307 General Hospital of the People's Liberation Army were collected and centrifuged at 1000 g for 10 min, washed three times, and then resuspended in phosphate-buffered saline (PBS, pH 7.4) to attain a 10% (v/v) erythrocyte suspension. Next, 50 μ L of this suspension was incubated with 200 μ L of two-fold serial dilutions (7.8–1000 μ g/mL) of sample solution in PBS at 37 °C for 1 h. After centrifugation, the supernatant absorbance at 540 nm was measured by an M5 SpectraMax® M5 spectrophotometer (Molecular Devices, USA). The hRBCs in PBS and 0.1% Triton X-100 were regarded as negative and positive controls, respectively. The tests were performed in triplicate, and the mean values of three independent measurements were employed for data analysis. The percentage of hemolysis was calculated as follows: percentage of hemolysis = $(A_{\text{compds}} - A_{\text{PBS}}) / (A_{\text{TritonX-100}} - A_{\text{PBS}}) \times 100\%$.

4.6 Cytotoxicity assay

Cytotoxicity of the compounds was assessed against MCF-7 cells and mouse fibroblast 3T3 cells. Briefly, the cells were respectively seeded in 96-well cell culture plates (Corning, Inc., Corning, NY) at a density of approximately 1.0×10^4 cells per well in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum and incubated for 24 h. The cells were washed twice with PBS and incubated with samples (100 μ L) in dimethyl sulfoxide diluted with DMEM at the indicated concentration at 37 °C for 6 h. The cells were washed with PBS to remove the peptides and resuspended in PBS (80 μ L). Thereafter, 20 μ L of Cell Titer 96® AQueous One Solution Reagent (Promega Corp., Madison, WI, USA) was added to each well, followed by an incubation period of 1 h. The solution absorbance was measured at 540 nm using a SpectraMax® M5 spectrophotometer (Molecular Devices, USA). PBS was used as a negative control. Cell viability data were obtained by calculating the ratio of viable cells in the treated cultures to the untreated control cells. The experiment was repeated three times for each concentration.

4.7 Susceptibility of plasma

The BAc-R3 and MSI-78 were dissolved in water, sonicated and centrifuged. A 200 μ L of the above solutions were mixed with 800 μ L of plasma, and then the mixtures were incubated at 37 °C respectively. At different time points (0 min, 1 min, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 6 h, 12 h and 18 h), 40 μ L of samples were removed and quenched with 40 μ L 5 % TFA/CH₃CN. The samples were centrifuged (5 min, 10000 g), and the supernatant was analyzed by HPLC.

4.8 Antibacterial activity in plasma

The sensitivity of the compounds to degradation in plasma was determined by testing the inhibition range after the compounds were incubated with human plasma for different periods of time. Briefly, 0.5 mL of fresh human plasma was added into a 1.5-mL centrifuge tube containing 0.5 mL of saline solution (0.9% NaCl). Then, the compounds at a concentration of 1 mg/mL were incubated at 37 °C for periods of 1 h, 3 h, 6 h, and 18 h. In parallel, sample solutions incubated with sterile broth in

the absence of plasma were regarded as the 0-h time point. After each incubation period, 100 μ L of sample solution was subjected to two-fold serial dilution in sterile broth in 96-well plates (Corning, Inc., Corning, NY), added to 10 μ L of the bacterial suspension, and incubated at 37 °C overnight. Next, the MIC values were determined as mentioned in the antibacterial assay. The results were determined from three independent experiments repeated three times.

4.9 Confocal laser scanning microscopy

Two different dyes, DAPI and PI, were used to assess the integrity of the bacterial membranes. *S. aureus* ATCC 25923 at the exponential growth phase was adjusted to 1.0×10^7 CFU/mL with sterile broth. Then, the bacteria were incubated with samples at $4 \times \text{MIC}$ at 37 °C for 2 h with agitation. The cells were centrifuged at 9000 g for 5 min in a microcentrifuge tube. The supernatant was removed, and the pellet was washed with PBS (pH 7.4) three times and then incubated with PI (10 μ g/mL) for 15 min at 4 °C in the dark. The excess PI was removed by washing twice with PBS (pH 7.4). Then, the cells were incubated with DAPI (20 μ g/mL) for 15 min at 4 °C in the dark, washed, and finally resuspended in PBS (pH 7.4). Bacteria without treatments were used as controls. The suspensions were pipetted into 15-mm glass bottom dishes and analyzed by confocal microscopy (LSM 510 META, Carl Zeiss, Germany).

4.10 TEM

TEM (Hitachi H-7650 microscope, Japan) was carried out to observe the morphological changes of microorganisms before and after treatment with compounds as reported.^[23] Briefly, *S. aureus* ATCC 25923 was cultured overnight to the exponential growth phase and then incubated with $4 \times \text{MIC}$ of compound for 2 h at 37 °C. Meanwhile, cells treated with PBS were regarded as controls. After treatment, the bacterial suspensions were centrifuged at 5000 g for 10 min, the supernatants were removed, the pellets were mixed with PBS (pH 7.4), and the mixture was centrifuged again to remove the PBS. Then, the microorganisms were fixed overnight with 3% glutaraldehyde solution in PBS (0.075 M, pH 7.4) at 4 °C, washed several times with PBS (0.075 M, pH 7.4), and post-fixed with 1% osmic acid in PBS (0.24 M, pH 7.4) for 1 h. After washing three times with PBS (0.075 M, pH 7.4) at 4 °C, the fixed samples were dehydrated with a series of graded ethanol and acetone solutions, incubated with a mixture of acetone and Epon812 resin (1:1, v/v) for 30 min at 4 °C, and then incubated with Epon812 resin overnight. Next, the samples were embedded in Epon812 resin, and ultrathin sections were obtained (60–70 nm) with an ULTRACUTS Ultramicrotome (RMC, USA). Lastly, the sections were stained with uranyl acetate and lead citrate for 10 min, respectively, before observation by TEM.

Acknowledgments

This research was supported, in part, by grants from the National Science Foundation of China (81373266 and 81573266) and a National Science and Technology Major Project of China grant (2012ZX09301003 and 2013ZX09102-010).

References and notes

1. Muffler, K.; Leipold, D.; Scheller, M. C.; Haas, C.; Steingroewer, J.; Bley, T.; Neuhaus, H. E.; Mirata, M. A.; Schrader, J.; Ulber, R. *Process Biochem.*

2011, 46, 1.

2. Dzubak, P.; Hajduch, M.; Vydra, D.; Hustova, A.; Kvasnica, M.; Biedermann, D.; Markova, L.; Urban, M.; Sarek, J. *Nat. Prod. Rep.* **2006**, 23, 394-411.
3. Fontanay, S.; Grare, M.; Mayer, J.; Finance, C.; Duval, R. E. *J. Ethnopharmacol.* **2008**, 120, 272.
4. Shai, L. J.; McGaw, L. J.; Aderogba, M. A.; Mdee, L. K.; Eloff, J. N. *J. Ethnopharmacol.* **2008**, 119, 238.
5. Perumal, Y.; Dharmarajan, S. *Curr. Med. Chem.* **2005**, 12, 657(10).
6. Wozniak, L.; Skapska, S.; Marszalek, K. *Molecules.* **2015**, 20, 20614.
7. Mei-Chin, Y.; Ming-Cheng, L.; Mei-Chin, M.; Chia-Yu, L. *J. Agr. Food Chem.* **2012**, 60, 7697.
8. Koren, E.; Torchilin, V. P. *Trends Mol. Med.* **2012**, 18, 385.
9. Dana Maria, C.; Kent, L.; Elo, E.; ülo, L. *Acs Nano.* **2014**, 8, 1972.
10. Ramsey, J. D.; Flynn, N. H. *Pharmacol. Therapeut.* **2015**, 154, 78.
11. Yong, H. N.; Park, I. S.; Hahm, K. S.; Song, Y. S. *J. Pept. Sci.* **2011**, 17, 812.
12. Bahnsen, J. S.; Franzyk, H.; Sandberg-Schaal, A.; Nielsen, H. M. *Biochim. Biophys. Acta.* **2013**, 1828, 223.
13. Oh, D.; Sun, J.; Nasrolahi Shirazi, A.; LaPlante, K. L.; Rowley, D. C.; Parang, K. *Mol. Pharm.* **2014**, 11, 3528.
14. Futaki, S. *Adv. Drug Deliv. Rev.* **2005**, 57, 547.
15. Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, 276, 5836.
16. Rothbard, J. B.; Garlington, S.; Lin, Q.; Kirschberg, T.; Kreider, E.; Mcgrane, P. L.; Wender, P. A.; Khavari, P. A. *Nat. Med.* **2000**, 6, 1253.
17. Christof, S.; Nirupam, P.; Beata, K.; Martin, G.; Borko, A.; Kai, M.; Dieter, S.; Faustin, K. I. *Antimicrob. Agents Chemother.* **2013**, 57, 4689.
18. Guzman, F.; Marshall, S.; Ojeda, C.; Albericio, F.; Carvajal-Rondanelli, P. *J. Pept. Sci.* **2013**, 19, 792.
19. Michael, Z. *Nature.* **2002**, 415, 389.
20. Wimley, W. C. *Acs Chem. Biol.* **2010**, 5, 905.
21. Chukung, A. F.; Bozzelli, K. N.; Lockwood, N. A.; Haseman, J. R.; Mayo, K. H. *Bioconjugate Chem.* **2004**, 15, 530.
22. Makovitzki, A.; Avrahami, D.; Shai, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, 103, 15997.
23. Liu, L.; Xu, K.; Wang, H.; Tan, P. K.; Fan, W.; Venkatraman, S. S.; Li, L.; Yang, Y. Y. *Nat. Nanotechnol.* **2009**, 4, 457.
24. Wang, C.; Lu, L.; Na, H.; Li, X.; Wang, Q.; Jiang, X.; Xu, X.; Yu, F.; Zhang, T.; Li, J.; Zhang, Z.; Zheng, B.; Liang, G.; Cai, L.; Jiang, S.; Liu, K. *J. Med. Chem.* **2014**, 57, 7342.
25. Parra, A.; Martin-Fonseca, S.; Rivas, F.; Reyes-Zurita, F. J.; Medina-O'Donnell, M.; Rufino-Palomares, E. E.; Martinez, A.; Garcia-Granados, A.; Lupianez, J. A.; Albericio, F. *ACS Comb. Sci.* **2014**, 16, 428.
26. Chu, F.; Xu, X.; Li, G.; Gu, S.; Xu, K.; Gong, Y.; Xu, B.; Wang, M.; Zhang, H.; Zhang, Y.; Wang, P.; Lei, H. *Molecules.* **2014**, 19, 18215..
27. Chan, D. I.; Prenner, E. J.; Vogel, H. J. *Biochim. Biophys. Acta.* **2006**, 1758, 1184.
28. Hein, J. E.; Fokin, V. V. *Chem. Soc. Rev.* **2010**, 39, 1302.
29. Ge, Y.; Macdonald, D. L.; Holroyd, K. J.; Thornsberry, C.; Wexler, H.; Zasloff, M. *Antimicrob. Agents Chemother.* **1999**, 43, 782.
30. Hilchie, A. L.; Wuerth, K.; Hancock, R. E. *Nat. Chem. Biol.* **2013**, 9, 761.
31. Scott, R. W.; DeGrado, W. F.; Tew, G. N. *Curr. Opin. Biotech.* **2008**, 19, 620.
32. Radziszewsky, I. S.; Rotem, S.; Bourdetsky, D.; Navon-Venezia, S.; Carmeli, Y.; Mor, A. *Nat. Biotechnol.* **2007**, 25, 657.
33. Konai, M. M.; Chandradhish, G.; Venkateswarlu, Y.; Sandip, S.; Jayanta, H. *J. Med. Chem.* **2014**, 57, 9409.
34. Made, V.; Els-Heindl, S.; Beck-Sickinger, A. G. *Beilstein J. Org. Chem.* **2014**, 10, 1197.