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ARTICLE

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Linearized teixobactin is inactive and after sequence enhancement, kills methicillin-resistant *Staphylococcus aureus* via a different mechanism

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Abstract

Staphylococcus aureus (*S. aureus*) is a highly adaptable pathogen that can rapidly develop resistance to conventional antibiotics such as penicillin. Recently, teixobactin was discovered from uncultivated soil bacteria by using the i-chip technology. This depsipeptide forms an ester bond between the backbone C-terminal isoleucine carboxylic acid and the hydroxyl group of threonine at position 8. Also, it contains multiple nonstandard amino acids, making it costly to synthesize. This study reports new peptides designed by linearizing teixobactin. After linearization and conversion to normal amino acids, teixobactin lost its antibacterial activity. Using this inactive template, a series of peptides were designed via hydrophobic patching and residue replacements. Three out of the five peptides were active. YZ105, only active against Gram-positive bacteria, however, showed the highest cell selectivity index. Different from teixobactin, which inhibits cell wall synthesis, YZ105 targeted the membranes of methicillin-resistant *S. aureus* (MRSA) based on kinetic killing, membrane permeation, depolarization, and scanning electron microscopy studies. Moreover, YZ105 could kill nafcillin-resistant MRSA, Staphylococcal clinical strains, and disrupted preformed biofilms. Taken together, YZ105, with a simpler sequence, is a promising lead for developing novel anti-MRSA agents.

KEYWORDS
antibiofilm, antimicrobial peptide, mechanism of action, methicillin-resistant *Staphylococcus aureus*, peptide design

1 | INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a high-priority pathogen listed by World Health Organization.[1] *S. aureus* can cause numerous diseases, including pneumonia, sepsis, skin, soft tissue, bloodstream, and surgical site infections. *S. aureus* and *Clostridium difficile* cause over 25,000 deaths per year.[2,3] *S. aureus* is a highly adaptable pathogen that can rapidly develop resistance to conventional antibiotics. MRSA strains have been isolated in both communities and hospitals. This pathogen utilizes numerous virulence factors to facilitate infections and biofilm formation provides yet another mechanism for resistance to both antibiotics and immune response.[4–6]

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Host defense antimicrobial peptides (AMPs) have been recognized as one of the most promising candidates for developing new types of antimicrobials to combat antibiotic-resistant pathogens.[7–10] According to the AMP database (APD; https://aps.unmc.edu), over 3000 natural AMPs have been isolated and characterized from six life kingdoms, including bacteria, archaea, protists, fungi, plants, and animals.[11–13] Many of these peptides show a broad activity spectrum and are able to kill bacteria, fungi, viruses, and parasites. The majority (87%) of the AMPs are short with less than 50 amino acids. They are usually positively charged (net charge +1–+7) and hydrophobic (20%–70%).[14] Moreover, recent bioinformatics analysis of over 1000 amphibian AMPs reveals a decrease in hydrophobic content but an increase in net charge when the peptide length rises.[15] Such an amphipathic feature enables them to rapidly recognize anionic bacteria for killing. It is believed that membrane targeting is a major reason for the lasting potency of AMPs. This important feature, plus the successful examples of AMPs in clinical use, has stimulated extensive research interest in AMPs.[7–10,16]

To identify potent antimicrobials against MRSA, both library and structure-based approaches are useful.[16] The discovery of the bacterial 70S ribosome as the target for proline-rich peptides[17,18] paved the way for classical structure-based design of novel AMPs.[19] There are also non-classical approaches. Based on the NMR spectral differences of membrane-bound and unbound peptide regions (i.e., NMR-trim technology), Wang and colleagues identified the major antimicrobial region (residues 17–32) of human cathelicidin LL-37.[20] On the basis of the 3D structure, this peptide was then successfully engineered into selective, stable and potent peptides (e.g., 17BIPHE2) against the ESKAPE pathogens, including *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Enterobacter* spp.[21] While multiple laboratories explored peptide design or surface coating based on KR-12,[22–26] the minimal antibacterial peptide of human LL-37,[27] we designed shorter peptides (C10-KR8) by conjugating KR-12 fragments with fatty acid chains with various lengths.[28] Library screening was also utilized to identify new candidates.[29,30] As a different approach, representative natural peptides from the APD were screened.[31,32] APD also enabled the development of other methods, including more recent machine learning predictions.[33,34,35] While Loose *et al.* published the Grammar approach,[36] we developed the database filtering technology for designing novel peptides. Different from the broad-spectrum 17BIPHE2 peptide, DFTamP1, the first helical peptide designed in this manner, is a narrow-spectrum peptide as it is active against Gram-positive *S. aureus*, but did not kill Gram-negative bacteria *Escherichia coli* (*E. coli*), *P. aeruginosa*, and Gram-positive *Bacillus subtilis*.[37] It is remarkable that all the AMPs in the APD show a linear correlation between averaged hydrophobic and arginine contents.[38] Based on this relationship as well as the idea of low cationicity,[39] a short amphipathic peptide, horine, was also designed and found to be primarily active against Gram-positive pathogens such as MRSA.[38]

Interestingly, teixobactin, discovered by the i-chip technology, is primarily active against Gram-positive pathogens as well.[40] These include *S. aureus* (MSSA and MRSA), *Enterococcus faecalis* (VRE), *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Bacillus anthracis*, *Clostridioides difficile*, *Propionibacterium acnes*, and *Mycobacterium tuberculosis* H37Rv (MIC 0.005–0.25 μg/mL). Teixobactin, however, has a different construction where an N-terminal linear sequence is followed by a cyclic structure achieved via a chemical bond between T8 hydroxyl and the carboxylic group of I11. In addition, teixobactin contains multiple non-standard amino acids, conferring structural and functional properties to the peptide.[41] However, such an extensive post-translational modification makes it costly to chemically synthesize this peptide in laboratories. Consequently, additional research efforts are spent to advance its development.[42] Total synthesis of teixobactin has been reported.[43,44] Zhang *et al.* screened aqueous solubility of teixobactin analogs to reject candidates with a propensity for aggregation and gel formation.[45] This is because a lack of solubility may limit the formulation process and its clinical use, while the formation of amyloid-like fibrils could produce unwanted cytotoxicity. Alanine scan of teixobactin reveals a possible change of position 3 to other amino acids without losing activity and the cationic residue at position 10 is not necessary for activity.[46]

This study deviates from the above efforts by exploiting a new avenue to peptide discovery. It came into our attention that the amino acid sequence of teixobactin is also amphipathic, similar to our database designed peptide DFTamP1. We were curious whether the cyclic peptide chain of teixobactin could be opened as a template for us to design new anti-MRSA peptides. Specifically, we wanted to address the following questions: (1) Will the linearized form of teixobactin be antimicrobial? (2) If yes, what is the activity spectrum? (3) If not, can we make the peptide antimicrobial? (4) Will the designed peptide have a different activity spectrum? (5) Will the linearized peptide work in the same mechanism as teixobactin? Our study led to new AMPs. Here we report our results.

2 | MATERIALS AND METHODS

2.1 | Peptide and chemicals

All peptides were chemically synthesized and purified to >95% (Genemed Synthesis, TX). The quality of each peptide was determined based on Mass Spectrometry and HPLC (See Supporting Figure S1). Peptides stock solutions were freshly made by solubilizing them in autoclaved distilled water. Since there is no tryptophan in each sequence, peptide concentrations were quantitated using UV spectroscopy based on the Waddel method.[47] Other chemicals were purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma (MO, USA) unless specified.

2.2 | High-performance liquid chromatography retention time measurements

To confirm peptide quality and to measure the retention time, each peptide was injected into an analytical reverse-phase WATERS C8 symmetry column (3.9 × 150 mm) equipped on a Waters High-performance liquid chromatography (HPLC) system. The peptide detected at

220 nm was eluted with a gradient of acetonitrile (containing 1% TFA) from 5% to 95% at a flow rate of 1 ml/min.

2.3 | Antimicrobial assays

The IBC protocol for the use and storage of bacteria for the antimicrobial studies in the Wang lab was approved by the University of Nebraska Medical Center (UNMC). This protocol is renewed every year. All personnel involved are properly trained and approved before handling any bacteria. Antimicrobial assays were conducted in a BSL-2 biosafety hood. Multiple bacterial strains were employed in this study: Gram-positive methicillin-resistant *S. aureus* USA300 LAC, *Staphylococcus epidermidis* 1457, and Gram-negative bacteria *Escherichia coli* E423-17 (ampC), *P. aeruginosa* E411-17, *K. pneumoniae* E406-17, and *A. baumannii* B28-16. In addition, several clinical strains of *S. aureus* were tested. Bacteria were cultivated in tryptic soy broth (TSB) from BD Bioscience MD, USA. We found identical results previously in TSB and Mueller–Hinton broth.[28]

The antibacterial activity of peptides was evaluated using a standard broth microdilution protocol with minor modifications.[39,48] In brief, logarithmic phase bacterial cultures (i.e., optical density at 600 nm ≈ 0.5) were diluted to 0.001 OD and aliquoted 90 μl per well into a 96-well polystyrene microplate containing 10 μl of serially diluted peptides. The plates were incubated at 37 °C overnight for 18–20 h and were then read at 630 nm using a ChroMate Microplate Reader (GMI, Ramsey, MN). The minimal inhibitory concentration (MIC) was the lowest peptide concentration that fully inhibited bacterial growth.

To study the influence of medium conditions on the antimicrobial activity of peptides against *S. aureus* USA300, the TSB medium condition - was changed by adjusting pH to 5.5, 6.8, 7.4, and 8.0, by adding an aliquot of sodium chloride (NaCl) stock solution to 100 mM, or by adding human serum to 1, 5, and 10%.

2.4 | Hemolytic assay

The IRB requirement for the use of human red blood cells for toxicity evaluation was waivered by the UNMC. Hemolytic assays of peptides were performed as described.[39] Briefly, human red blood cells (hRBCs), obtained from the UNMC Blood Bank, were washed three times with isotonic saline (0.9% NaCl) until the supernatant is clear. The washed cells were then diluted to 2% (v/v). To each well containing serially two-fold diluted peptides (10 μl), 90 μl of hRBCs were added and incubated at 37 °C for 1 h. Post incubation, the plates were spun at 2000 rpm for 10 min, aliquots of the supernatant were carefully transferred to a clean 96-well microplate. The amount of cells lysed is proportional to the hemoglobin released and measured at 545 nm using a ChroMate microplate reader. The percent lysis was calculated by assuming 100% release when human blood cells were treated with 1% Triton X-100, and 0% release when incubated with PBS. The peptide concentration that caused 50% lysis of hRBCs is defined as HC_{50}.

2.5 | Killing kinetics of exponential bacteria

Killing experiments were conducted by taking aliquots of cultures (~10^5 CFU) of *S. aureus* USA300 treated with YZ105 or antibiotics at 5, 15, 30, 60, and 120 min. After dilution 100-fold, the culture was plated on Luria-Bertani agar plates and incubated at 37 °C overnight. The bacterial colonies were enumerated next day in the morning.

2.6 | Real time fluorescence-based membrane integrity

The experiment was performed as described previously with minor modifications.[38,49] Serially diluted 10× peptides (10 μl each well) were created in 96-well microtiter plates. Propidium iodide (PI) (2 μl) at a fixed concentration of 20 μM was added to each well followed by 88 μl of the *S. aureus* USA300 culture (a final OD_{600} ~ 0.1 in PBS). The plate was incubated at 37 °C with continuous shaking at 100 rpm in a FLUOstar Omega (BMG LABTECH, NC, USA) microplate reader. The sample fluorescence was read every 5 min for 24 cycles with excitation and emission wavelengths of 584 nm and 620 nm, respectively. Plots were generated using average values from the experiments using GraphPad Prism.

2.7 | Membrane depolarization of bacteria

An overnight culture of *S. aureus* USA300 was grown in TSB to the exponential phase. Cells were centrifuged, washed 2× with PBS, and resuspended in twice the volume of PBS containing 25 mM glucose and incubated at 37 °C for 15 min. For membrane depolarization measurements, the dye DiBAC4 (3) bis-(1, 3-dibutylbarbituric acid) trimethine oxonol (ANASPEC, CA, USA) was added to a concentration of 500 nM and vortexed gently.[48] Aliquots of 90 μl of the energized bacteria solution were loaded to the plate (Corning COSTAR), which was fed into a FLUOstar Omega (BMG LABTECH Inc., NC, USA) microplate reader. Fluorescence was read for 20 min at excitation and emission wavelengths of 485 nm and 520 nm, respectively, to get dye normalization. Then 10 μl of peptide solutions were added, gently mixed. Fluorescence reads were recorded for 40 min, where Triton X-100 (0.1%) was used as a positive control.

2.8 | Scanning electron microscopy

For scanning electron microscopy (SEM), *S. aureus* USA300 was treated with 2× MIC peptide and fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS provided by the EM core lab. Further, the samples were washed with Sorensen's Buffer with three changes, 5 min each change. Samples were post-fixed in 1% osmium tetroxide in water for 30 min. After post-fixation, all samples were washed in Sorensen's buffer with three changes at 5 min each change. All samples were dehydrated through a graded ethanol series (50%, 70%, 90%, 95%, 100% × 3 changes) for 10 min each dehydration step. Subsequently, samples were placed in

HMDS 100% for 10 min for 3 changes and left in HMDS in open dishes in the fume hood overnight to allow the HMDS to evaporate. The following day the samples were mounted on 25 mm aluminum SEM stubs with carbon adhesive tabs. Silver paste was placed around the edges of the samples. Samples were Sputter Coated with 50 nm of gold/palladium in a Hummer VI Sputter Coater (Anatech Ltd.) and examined in a FEI Quanta 200 SEM operated at 25 Kv using the EM Core Facility on campus.

2.9 | Effects of peptides on established biofilms

Antibiofilm potency of the peptide against biofilms was evaluated against MDR as reported.[50,51] Briefly, *S. aureus* USA300 LAC was prepared from an exponential phase culture. Each well of the microtiter plate (Corning Costar Cat No. 3595) was aliquoted with 180 μl of inoculum and the plates were incubated at 37 °C for 24 h to form biofilms. Culture treated with water served as the positive control while media without bacterial inoculation served as the negative control. Media were aspirated post incubation and the attached biofilms were washed with 1× PBS to remove the planktonic bacteria. Each well was aliquoted with 20 μl of 10× peptide solution and 180 μl of fresh TSB media, and plates were further incubated at 37 °C for 24 h. Media were aspirated from the wells and washed with 1× PBS to remove planktonic cells. Live cells in the biofilms were quantitated using the XTT [2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2H-tertazolium-5-carboxanilide] assay by following the manufacturer's instructions with modifications. The 180 μl of fresh TSB and 20 μl of XTT solution were added to each well and the plates were further incubated at 37 °C for 2 h. Absorbance was read at 450 nm (only media with XTT containing wells served as the blank) using a ChromateTM microtiter plate reader. Biofilm growth percent after peptide treatment was plotted relative to that without treatment (100%). The data were represented as mean ± SD, the significance at $p < 0.05$. Plots were generated using GraphPad prism.

3 | RESULTS

3.1 | Peptide design, antibacterial activity and hemolytic toxicity

To generate the teixobactin template, the ester bond was broken to make the peptide linear. A series of peptides were designed and the peptide quality data are provided in Figure S1. The calculated sequence properties of these peptides are listed in Table 1 (chemical structure in Figure S2). To validate peptide purity, we also obtained the HPLC elution profiles for all the peptides on our Waters HPLC system (Figure S3). Our design started from converting all nonstandard amino acids to normal ones. Thus, the N-terminal methyl was eliminated and enduracididine 10, a cyclic analog of arginine, was converted to arginine. We also used L-amino acids to replace the four D-amino acids. This led to peptide YZ103 (amino acid sequence in Table 1). YZ103, however, was not antimicrobial against any bacteria till the highest concentration we tested (Table 2). To enhance peptide activity, we first attached a pair of leucines to the C-terminus. Again, the leucine-patched peptide YZ104 (Table 1) was unable to inhibit the growth of a panel of Gram-positive and Gram-negative bacteria. Because leucine is proved to be important in MRSA killing,[37] we made three changes in YZ104: I2L, I6L, and A9L and the resultant peptide was named YZ105. This peptide displayed antibacterial activity against two *Staphylococcal* strains (*S. aureus* and *S. epidermidis*), but not Gram-negative pathogens in Table 2. This peptide is rather potent as its MIC (1 μM) is lower than other anti-MRSA peptides we obtained

TABLE 1 Amino acid sequences and calculated properties of designed peptides

Peptide	Amino acid sequence[a]	Length	Net charge	Pho	Boman index (kcal/mol)	GRAVY
YZ103	FISQIISTARI	11	+2	55%	0.48	1.12
YZ104	FISQIISTARI**LL**	13	+2	62%	−0.34	1.53
YZ105	F**L**SQI**L**STLRI**LL**	13	+2	62%	−0.58	1.57
YZ106	F**L**S**K**I**L**STLRI**LL**	13	+3	62%	−0.58	1.55
YZ107	F**L**S**K**I**L**STLRI**LF**	13	+3	62%	−0.43	1.47

[a]C-terminal amidated. Appended or changed amino acids are in bold. Peptide parameters were calculated using the APD tool: https://aps.unmc.edu/prediction/predict. Pho is defined as the hydrophobic content of the peptide where residues I, L, A, M, V, C, W, and F are hydrophobic.[11,12]

TABLE 2 Antibacterial minimal inhibitory concentrations (μM) of teixobactin-derived peptides

Peptide	*Staphylococcus aureus* USA300 LAC	*Staphylococcus epidermidis*1457	*Escherichia coli* E423-17	*Acinetobacter baumannii* B28-16	*Pseudomonas aeruginosa* E411-17	*Klebsiella pneumoniae* E406-17
YZ103	>16	>16	>16	>16	>16	>16
YZ104	>16	>16	>16	>16	>16	>16
YZ105	1	1	>16	>16	>16	>16
YZ106	2	1–2	8–16	8	>16	>16
YZ107	2	2	8	4–8	8–16	>16

previously.[21,28,37,38,39] The increased activity of YZ105 might be attributed to its increased hydrophobicity as indicated by a longer retention time on the reverse-phase HPLC column (Table 3). We then measured the hemolytic toxicity of this peptide. YZ105 showed poor hemolysis with a 50% hemolytic concentration at 150 μM. This led to a cell selectivity (HC_{50}/MIC = 150/1) index of 150 (Table 3). We also explored whether there was room to further increase peptide activity. Since basic amino acids are known to be important in AMP design, we replaced Q4 with K. The resulting peptide YZ106 gained activity against Gram-negative bacteria *E. coli* and *A. baumannii* (MIC 8–16 μM). This is consistent with the fact that positively charged amino acids are more important for AMP activity against Gram-negative pathogens.[39,52] This basic amino acid in YZ106 led to a reduced HPLC retention time compared to YZ105. Finally, a conservative mutation of L13 to F13 in YZ107 did not increase the peptide activity. Unfortunately, these two peptides also showed increased hemolytic toxicity: YZ106 (HC_{50} 50 μM) and YZ107 (HC_{50} 25 μM). Because cell selectivity indexes dropped, these alterations were undesirable (Table 3). Hence, we chose YZ105 hereinafter for additional studies.

3.2 | Antimicrobial robustness, killing kinetics, and antibiofilm activity

Because media conditions can influence antimicrobial activity of AMPs such as LL-37, we compared the activity of YZ105 in the presence of salts and human serum or at different pH values. Under a normal condition in TSB, pH 7.4, YZ135 showed an MIC value in the range of 1–2 μM. It became more active at pH 8. At pH 6.8, it had a similar MIC of 2 μM. A further decrease in pH to 5.5 reduced the peptide activity by 2–4 fold (Table 4). The addition of 100 mM NaCl did not affect the activity of YZ105. With an increase in human serum from 1%, 5%, to 10%, there was an increase in MIC to 8–16 μM, indicating a potential binding of the peptide to human serum proteins at high-serum conditions.[28] For comparison, we also included nafcillin (a narrow-spectrum beta-lactam antibiotic of the penicillin class) and linezolid (an antibiotic in the family of oxazolidinones). While pH had little effect on the activity of nafcillin, 100 mM NaCl showed a small effect and serum did increase its MIC values by 4–8 fold. Linezolid showed robust antibacterial activity against *S. aureus* USA300 by keeping similar MIC values under different pH and serum conditions, implying minimal association with serum proteins.

The antimicrobial activity of YZ105 was also tested against various *S. aureus* clinical strains (Table 5). YZ105 inhibited the growth of *S. aureus* USA200, USA300, USA400, Newman, and Mu50 equally well at 1–2 μM. It also killed nafcillin-resistant *S. aureus* USA300 at 2 μM (cf. an MIC of 1024 μM for nafcillin). In contrast, YZ103, a linearized analog of teixobactin, did not inhibit any of these *Staphylococcal* strains at 32 μM.

To gauge the killing ability of YZ105, MRSA killing kinetics was followed by colony counting. At 4 × MIC, it reduced MRSA counts by

TABLE 3 Hemolytic toxicity of YZ peptides

Peptide	Estimated HC_{50} (μM)	Cell selectivity index[a]	HPLC retention time (min)[b]
YZ103	>200	NA	8.770
YZ104	>200	NA	11.746
YZ105	150	150	13.945
YZ106	50	25	12.312
YZ107	25	12.5	12.297

[a]Defined as the ratio between MIC against *S. aureus* SA300 (Table 1) and HC_{50} of each peptide in this table.
[b]See Supporting information for HPLC elution profiles.

TABLE 4 Effects of pH, salt, and serum on anti-*staphylococcal* activity of YZ105 and antibiotics

Compound	pH 7.4	pH 8	pH 6.8	pH 5.5	100 mM NaCl	1% serum	5% serum	10% serum
YZ105	1–2	<0.5	2	4–8	2	2	8	8–16
Nafcillin	0.5–1	1	1	0.5	1–2	1	2–4	4
Linezolid	4	4–8	4	4	4	4	8	8

TABLE 5 Antibacterial assay of YZ105 on *Staphylococcus aureus* clinical strains

Peptide	USA200	USA300	USA400	Newman	Mu 50	Nafcillin-resistant USA300
YZ103	>32	>32	>32	>32	>32	ND[a]
YZ105	1	1	1	2	2	2

[a]Not determined. YZ103 served as a negative control.

2 logs in 2 h (Figure 1). In contrast, linezolid reduced CFU of *S. aureus* USA300 by 1 log, while nafcillin barely reduced bacterial burden relative to the untreated control. Hence, YZ105 was able to kill *S. aureus* more effectively than either nafcillin or linezolid.

We also investigated the effect of the peptide on 24 h preformed biofilms. YZ103, which was not antibacterial, did not show any effect on the preformed biofilms of *S. aureus* USA300 (not shown). YZ105, however, showed a dose-dependent effect on the biofilms of *S. aureus* USA300. It essentially eliminated the preformed biofilms at 4 μM or above (Figure 2a). In contrast, nafcillin did not display a clear effect (Figure 2b). It is notable that linezolid was able to reduce preformed biofilms of *S. aureus* in a dose-dependent manner (Figure 2c), although much less effective than YZ105. Interestingly, linezolid became more effective in the presence of 2 μM YZ105 (Figure 2d). These results illustrate an antibiofilm advantage by combining antibiotic linezolid with the newly designed peptide YZ105.

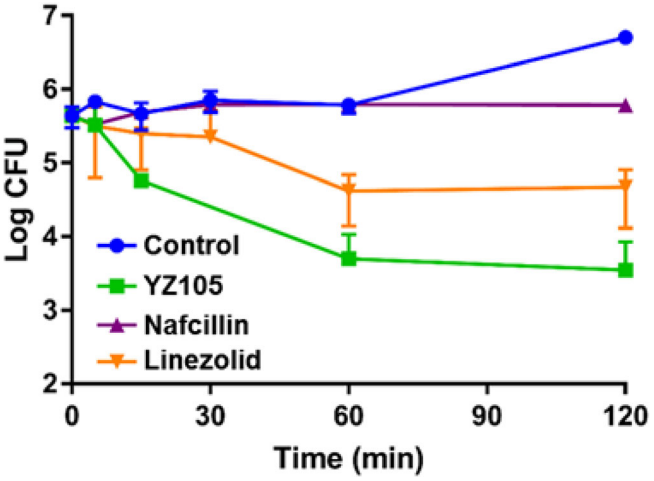

FIGURE 1 Killing kinetics of YZ105 against *Staphylococcus aureus* USA300 LAC. The killing was conducted in the presence of four fold minimum inhibitory concentration (MIC) of either the peptide (4 uM, green), nafcillin (2 μM, purple), or linezolid (16 μM, gold) using the exponential phase bacteria

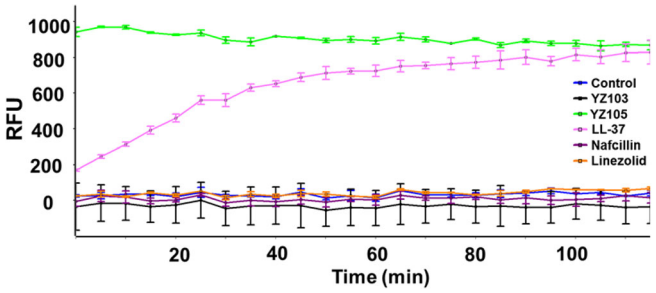

FIGURE 3 Membrane permeabilization of *Staphylococcus aureus* USA300 in PBS by YZ103 (16 μM), YZ105 (4 μM), nafcillin (2 μM), linezolid (16 μM), and LL-37 (16 μM), all at 4 × MIC, in the presence of 20 μM propidium iodide. YZ105 (green line) is even more powerful than LL-37 (pink line)

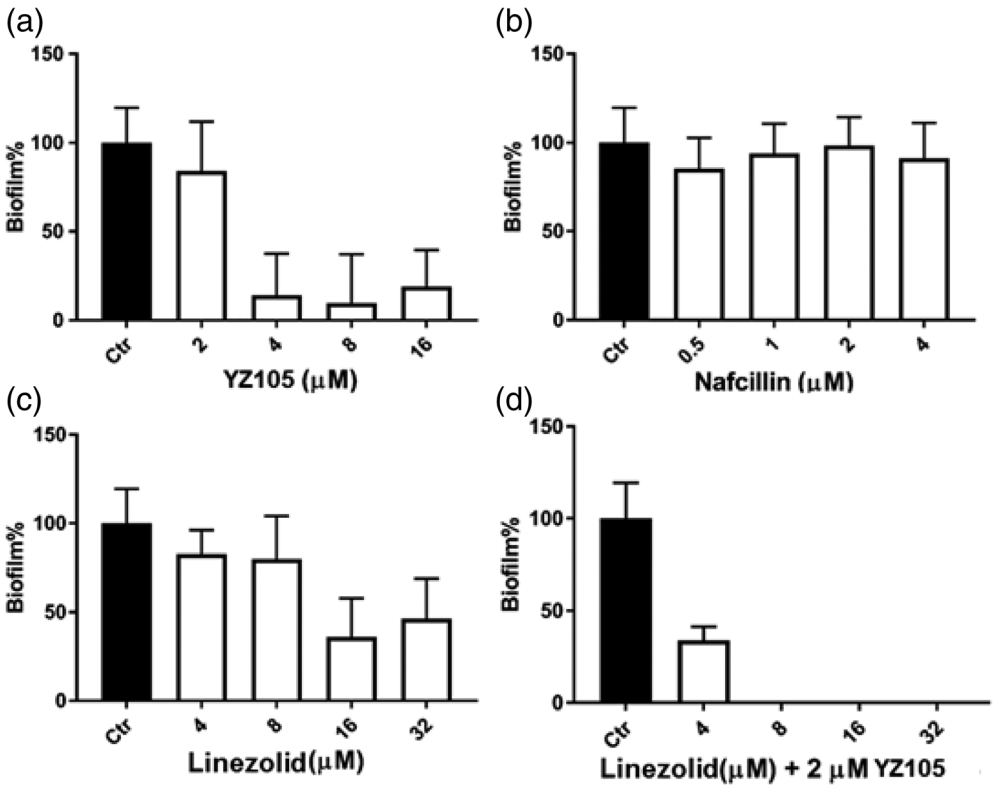

FIGURE 2 Antibiofilm activity of YZ105 (a), nafcillin (b), linezolid alone (c) and in combination (d) against *Staphylococcus aureus* (*S. aureus*) USA300. Biofilms of *S. aureus* were formed in 24 h

3.3 | Mechanism of action

To get insight into the antimicrobial mechanism, we then investigated the membrane permeabilization of the peptide. While no clear fluorescence increase was observed for the untreated MRSA, fluorescence rapidly increased in the presence of the non-membrane permeable dye PI when treated with YZ135. It appeared that the effect of YZ105 (Figure 3 green line) was much stronger than that of human cathelicidin LL-37, which showed steady increase in fluorescence with time (pink line in Figure 3). In contrast, YZ103 (Figure 3 black line), which was unable to inhibit the growth of *S. aureus* USA300, did not permeabilize the bacterial membranes since the curve was identical to that untreated (blue). Likewise, neither nafcillin nor linezolid permeabilized the membranes of *S. aureus*. This is consistent with the mechanisms of action of these antibiotics. While nafcillin targets the cell wall of *S. aureus*, linezolid inhibits protein synthesis by binding to ribosomal RNA of the 50S subunit.

AMPs may also perturb membrane potential. In our experiment setup, YZ105 was able to depolarize the membrane potential of *S. aureus* USA300 similar to the positive control Triton X-100 (Figure 4). In contrast, neither nafcillin nor linezolid was able to since the curves remained the same as that of untreated control.

To observe the effect of the peptide on bacteria, SEM was utilized. While *S. aureus* in the untreated control appeared smooth spheres, the surfaces of treated bacterial cells were clearly damaged (Figure 5), providing direct evidence for membrane disruption.

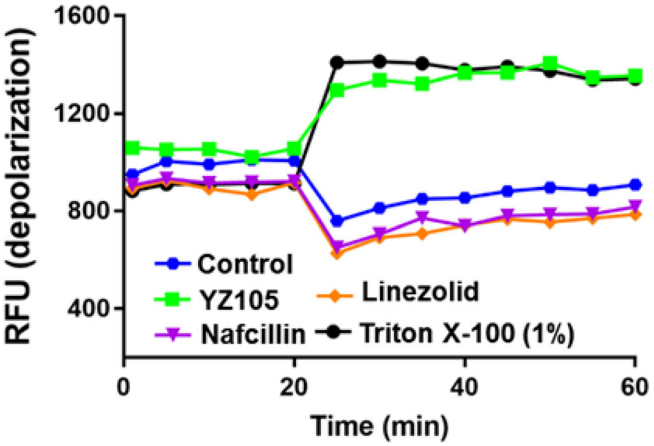

FIGURE 4 Membrane depolarization of *Staphylococcus aureus* USA300 by YZ105, nafcillin, linezolid and triton X-100 (1%). Nonmembrane targeting antibiotics were used as negative controls. Both nafcillin (4 μM) and linezolid (32 μM) were unable to depolarize the membranes, while YZ105 (8 μM, green curve) could polarize the membrane to the extent of 1% triton-X 100 (positive control, black line)

4 | DISCUSSION

AMPs are diverse but can be unified into four major classes: O, P, S, and L.[14] Class O peptides form a head-to-tail peptide bond (also known as UCBB). Class P peptides contain a covalent bond from the side chain of one amino acid to termini of the peptide backbone (UCSB). The third class, S, is well known, typically with disulfide bonds between different side chains (UCSS). Finally, class L peptides are most popular since they are usually linear, amphipathic and can form amphipathic helices (UCLL). These peptide classes differ in many ways. This study investigated how peptide activity and mechanism of action change when a class P peptide is converted to a class L peptide. Teixobactin, a class P peptide, contains a cyclic structure due to a bond formed between the side chain of T8 and the carboxylic C-terminus of I11.[40] This peptide is found to act on bacterial cell wall.[53–55] It appeared that the cyclic structure of teixobactin was important for its activity since the linearized counterpart (using normal amino acids) was inactive against a panel of bacteria we tested in Table 2.

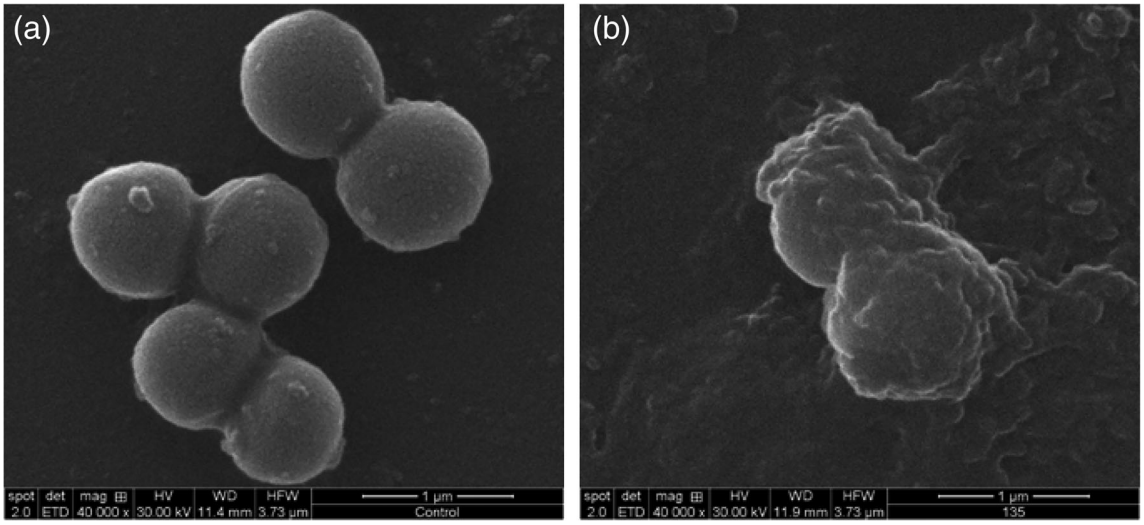

FIGURE 5 Scanning electron microscopic (SEM) images of *Staphylococcus aureus* USA300 in the absence (a) and presence (b) of peptide treatment with YZ105 at 4 μM

TABLE 6 Property comparison of teixobactin and YZ105

Peptide	Teixobactin	YZ105[a]
Class	P (UCBB)	L (UCLL)
Length	11	13
Net charge	+2	+2
Hydrophobic content	55%	62%
Amino acid type	Nonstandard and standard	Standard
Rich in certain amino acid (>25%)	Isoleucine (36%)	Leucine (38%)
Activity spectrum	Mainly G+	G+
Mechanism of action	Cell wall	Membranes

Abbreviations: G+: Gram-positive bacteria; Pho, hydrophobic content.
[a]Calculated in the APD (https://aps.unmc.edu).[12]

This observation would be in line with the crystal structural study of teixobactin analogs where all carbonyls of the ring residues orient on the same side, presumably for lipid II binding.[56] A rare residue L-allo-enduracidine at position 10 in the ring structure also enhances lipid II binding.[43] However, the peptide YZ105 became active only after both leucine patching and substitutions that made the peptide more hydrophobic than the converted teixobactin sequence (Table 1). These examples illustrate different sequence requirements for class P and class L peptides in killing MRSA. The requirement of a slightly longer sequence in the case of linear peptides is consistent with the bioinformatic analysis of the four unified classes of AMPs in the APD where the average length of class P is the shortest.[12] Since class P has shorter sequences, they may be useful templates to make new AMPs to combat drug-resistant pathogens.

Similar to teixobactin,[40] YZ105 kills Gram-positive *Staphylococcus* species but not Gram-negative bacteria (Table 2). To understand why YZ105 was primarily active against Gram-positive bacteria, we conducted a sequence analysis using the Calculation and Prediction interface of the APD (Table 1). YZ105 is highly hydrophobic with a hydrophobic ratio of 62% but low in basic amino acids (only one arginine in the sequence). Due to C-terminal amidation, YZ105 has a net charge of +2 (Table 1). Its sequence most resembles (61.5% similarity) frog temporin-1SPb[57] in the APD. The amphipathic nature of YZ105 is also similar (50% similarity) to our designed peptide DFTamP1.[37] The common feature of these peptides is high-hydrophobic ratios and low cationicity. Our recent study uncovered the importance of such a peptide composition for systemic efficacy against MRSA.[28,39] Since YZ105 has such a feature as well as a high-cell selectivity index (HC_{50}/MIC = 150), it deserves further studies. Note that YZ105 was synthesized using L-amino acids to facilitate the characterization reported here. This form is susceptible to proteases. Therefore, future studies may take advantage of a form of the peptide made of D-amino acids to gain stability to proteases and for better *in vivo* efficacy.

Mechanistically, teixobactin inhibits cell wall synthesis.[40] The cyclic structure of teixobactin plays a key role in cell wall binding.[56] In contrast, YZ105 kills *S. aureus* via a different mechanism by targeting membranes. We obtained multiple lines of evidence for membrane targeting. First, the peptide killed MRSA effectively (Figure 1). Second, YZ105 permeabilized the membranes of *S. aureus* (Figure 3). Third, it also depolarized the membrane potential of *S. aureus* (Figure 4). Finally, the membrane damage of YZ105 could be directly observed by SEM (Figure 5). Such a mechanistic difference is likely determined by the hydrophobic ratios of the peptides. YZ105, being more hydrophobic as evidenced by a higher hydrophobic content, more positive GRAVY value, more negative Boman index, and longer HPLC retention time than YZ103 (Table 1), is able to reach the membranes of MRSA. This is illustrated in the Graphic of this article for Table of Contents.

5 | CONCLUSION

Based on our observation of a similar amphipathic nature, we succeeded in converting teixobactin into a new anti-MRSA peptide. Some common and different features of teixobactin and YZ105 are summarized in Table 6. While teixobactin is a class P peptide (with a localized cyclic structure at the C-terminus), YZ105 belongs to class L (linear peptides). Teixobactin contains 36% isoleucine, while YZ105 contains 38% leucine. Teixobactin consists of numerous nonstandard amino acids which are important for structure and activity. In contrast, YZ105 comprises all standard amino acids, facilitating chemical synthesis for a detailed structure–activity relationship study. Despite these differences, both teixobactin and YZ105 share a similar activity spectrum and kill Gram-positive pathogens such as MRSA. However, they kill MRSA by different mechanisms. Teixobactin inhibits cell wall, while YZ105 causes damage to bacterial membranes. Taken together, we propose that YZ105 constitutes a useful candidate for further studies toward new antibiotic development.

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CONFLICT OF INTEREST

The authors do not declare conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw data are available upon request. Additional data can be found in the Supporting Information.

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