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Original article

Enhancement of the antimicrobial properties of KLR_{12} through charge addition and amino acid rearrangement

Noppasak Rojanasheeva[1,*], Paiboon Ngernmeesri[2], Chomdao Sinthuvanich[1]

[1]Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand
[2]Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand

Abstract

***Background**:* Understanding structure-function relationships is critical to the rational design of antimicrobial peptides with enhanced activity and specificity for therapeutic applications. In particular, α-helical peptides, known for their broad-spectrum antibacterial activity, can be modifying physicochemical properties such as charge, hydrophobicity, and amphipathicity.

***Objective**:* This study aims to enhance the antimicrobial potency of KLR_{12} by modifying its charge and amino acid rearrangement.

Methods: Native KLR_{12} and its derivatives, $KLR_{12\text{-}4R}$ and $KLR_{12\text{-}4R,7L}$, were designed and analysed for their physicochemical properties using prediction tools, APD3, BACHEM, HeliQuest, MACREL, QSPpred, and AMPfun. Circular dichroism (CD) spectroscopy was used to assess secondary structure. Antibacterial activity against *Staphylococcus aureus (S. aureus)* ATCC 25923 and *Escherichia coli (E. coli)* ATCC 25922 was determined by broth microdilution, and cytotoxicity in L929 cells was evaluated *in vitro*.

Results: Prediction scores indicated that all peptides have broad-spectrum potential with low cytotoxicity. Helical structures were observed in the presence of sodium dodecyl sulphate (SDS) solution. *In vitro* studies illustrated that the $KLR_{12\text{-}4R}$, exhibited twice the activity against *S. aureus* but was not effective against *E. coli* compared to native KLR_{12}. Additionally, $KLR_{12\text{-}4R,7L}$ demonstrated 16- and 4-fold higher activity against *S. aureus* and *E. coli*, respectively, compared to the native KLR_{12}. Furthermore, cytotoxicity in L929 cells was observed in a dose-dependent manner.

***Conclusion**:* Rational design of amphipathic KLR peptides led to enhanced antimicrobial potency. Among the three peptides, $KLR_{12\text{-}4R,7L}$ exhibited the highest potency. These peptides represent promising candidates for future drug development against infectious diseases.

***Keywords**:* Amino acid rearrangement, amphipathicity, antimicrobial peptides, charge addition.

***Correspondence to:** Noppasak Rojanasheeva,* Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
E-mail: noppasak.roja@ku.th

Antimicrobial peptides (AMPs) are the first host defence molecules secreted by Paneth cells to combat pathogenic microbes in infections. [1] Most importantly, they have been identified as alternative molecules to combat both general and drug-resistant bacteria, as their mechanism of action involves penetrating and disrupting the bacterial cell membrane. [2,3] However, several factors affect antimicrobial efficacy, including charge, hydrophobicity, amphipathicity, length, and aliphatic character. [4] Therefore, the rational design of AMPs has been one of the primary strategies to improve their effectiveness. [5] However, challenges such as peptide stability, potential resistance development, and maintaining low cytotoxicity must also be carefully considered to ensure their successful application. [6]

Designing AMPs can be categorised based on their structure, such as helix-based, hairpin-based, coil-based, and composite-based. [7] Additionally, each type has a distinct concept. The α-helical peptides are known for their broad-spectrum activity and potential for selective membrane disruption. The positively charged region interacts electrostatically with the negatively charged lipopolysaccharides on the outer membrane of Gram-negative bacteria and with the peptidoglycan of Gram-positive bacteria, while the hydrophobic part penetrates the membrane bilayer. [8] Moreover, the amphipathicity of α-helical AMPs is a major factor in the dispersive forces between charges and Van der Waals interactions. Several reports have shown that enhanced amphipathicity of α-helical AMPs can improve antibacterial activity [4,5,9]

KLR_{12} (KLLLRLRKLLRR) is a derivative of human β-defensin 3, generated by C-terminal truncation and amino acid substitutions that replace uncharged residues with hydrophobic ones. [10] It demonstrates high potential against both representative Gram-positive and Gram-negative bacteria, with MIC_{90} values of 16 and 4 μg/mL, respectively. Although these MIC_{90} values indicate good activity, the actual MIC_{100} values were approximately 128 and 64 μg/mL, respectively, with no available MBC data. Consequently, the antimicrobial properties of KLR_{12} can be enhanced through charge addition and amino acid rearrangement based on the α-helical structure.

In this study, we focused on α-helix-based AMPs and modified their sequences based on physicochemical property approaches. Several reports have highlighted factors that affect antimicrobial activity, including amphipathicity, charge, hydrophobicity, and aliphatic character. [2,4,5] For our study, we selected amphipathicity and charge as key factors in determining the antimicrobial properties of modified native KLR_{12}. This study aims to enhance the antimicrobial potency of KLR_{12} by modifying its charge and amino acid rearrangement.

Materials and methods

Peptide design and sequence analysis

The native KLR peptide was selected for modification through charge addition and amino acid rearrangement. To assess these modifications, sequence and structural properties were analysed using APD3 (https://aps.unmc.edu/prediction) and BACHEM (https://www.bachem.com/knowledge-center/peptide-calculator/) to calculate the physicochemical characteristics of the designed peptides. Helical wheel projections were generated using HeliQuest (https://heliquest.ipmc.cnrs.fr/HeliQuest_test.htm), while antimicrobial activity was predicted using MACREL (https://www.big-data-biology.org/software/macrel/), QSPpred (http://crdd.osdd.net/servers/qsppred/predictor.html), and AMPfun (http://fdblab.csie.ncu.edu.tw/AMPfun/run.html). Finally, KLR and its derivatives were synthesized using Fmoc chemistry by QYAOBIO (purity ≥ 90.0%, ChinaPeptides Co., Ltd., Shanghai, China). The purities of KLR_{12}, $KLR_{12\text{-}4R}$, and $KLR_{12\text{-}4R,7L}$ were 97.5 %, 98.8%, and 95.2%, respectively. The peptides were prepared by dissolving them in fresh ultrapure water to achieve a final concentration of 2 mM, followed by serial dilution as described in each assay.

Circular dichroism analysis

The secondary structure of each peptide in different environments were determined by a Jasco-815 spectropolarimeter using a 0.1-cm-path-length rectangular quartz cell. To investigate conformational changes of modified peptide in distinctive conditions, the peptides were prepared to final concentration of 150 μM in either 18.20 MΩ.cm water or 30 mM SDS. The mixtures were immediately incubated for 10 minutes at 25°C. Then, the ellipticity was measured from 190 to 260 nm at a scanning speed of 50 nm/min. The spectra were scanned three times for each condition. The percentage of secondary structures were calculated using BeStSel website (https://bestsel.elte.hu/).

Antimicrobial assay

The antimicrobial activity was evaluated against Gram-positive *(Staphylococcus aureus (S. aureus)* ATCC 25923) and Gram-negative (*Escherichia coli (E. coli)* ATCC 25922) bacteria. The MIC of each peptide was determined according to the Clinical and Laboratory Standards Institute (CLSI) M07-A10 guidelines, with a minimum of three independent biological replicates. Briefly, bacterial colonies were collected from Mueller-Hinton agar (MHA) and suspended in Mueller-Hinton broth (MHB). The suspension was adjusted to achieve an optical density (OD) at 600 nm of 0.100 ± 0.005, corresponding to a bacterial density of approximately 10^8 CFU/mL, which was then diluted to 10^6 CFU/mL. A 100 µL volume of a 2x peptide solution was prepared by performing a two-fold serial dilution of each peptide in a 96-well plate, with concentrations ranging from 1.56 to 200 µM. Then, 100 µL of the 10^6 CFU/mL bacterial suspension was added to each well, resulting in final peptide concentrations ranging from 0.78 to 100 µM. The plate was then incubated for 18 hours at 37 °C. The minimum inhibitory concentration (MIC) was defined as the lowest peptide concentration that prevented visible turbidity, as measured at 600 nm. Cultures without peptide and those treated with 5 mg/mL ampicillin in MHB were used as negative and positive controls, respectively. To determine the MBC, 2 µL of each sample at and above the MIC was cultured on MHA and incubated for another 24 hours at 37°C. The minimum bactericidal concentration (MBC) was defined as the lowest peptide concentration at which no colony growth was observed on the agar plates. The bacterial viability was assessed using the following equation:

Cell culture and cytotoxicity assay

The mouse fibroblast cell line (L929), at passage numbers ranging from three to nine, was cultured as a monolayer using DMEM-high glucose medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1.0% (v/v) penicillin/streptomycin. On the day of the experiment, 5×10^3 cells/well were seeded onto a 96-well plate and incubated in a humidified atmosphere of 5% CO_2 for 24 hours at 37°C. After removing the medium, 100 µL of the peptide solution ranging from 0.1 to 200 µM in DMEM-high glucose medium supplemented with 1% FBS, was added to each well and incubated under the same condition for further 24 hours. Cultures without peptides and those treated with 20.0% DMSO in 1.0% FBS DMEM-high glucose were used as negative and positive controls, respectively. After incubation, 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 2 hours to determine cell viability. The formazan crystals were then dissolved with DMSO, and absorbance was measured at 560 nm and 630 nm using a microplate reader. The experiment was conducted independently with at least four biological replicates. The viability was assessed using the following equation:

Results

Peptide design and sequence analysis

The KLR_{12} was used as a template for further modifications based on two key approaches, as shown in **Figure. 1A.** Firstly, the overall charge was increased by substituting the leucine residue at position four with arginine, resulted $KLR_{12\text{-}4R}$ with enhancing amphipathicity, increasing hydrophilicity from 50.0% to 58.0%, and raising the net charge from + 6 to + 7, as shown in **Table 1 and Figure 1B.** In addition, rearrangement was performed by switching leucine and arginine between positions four and seven, resulted in $KLR_{12\text{-}4R,7L}$. These changed further enhancing

Table 1. The physicochemical properties of native KLR and its derivatives.

Name	Amino acid sequences	MW (g/mol)	Net charge at pH 7	%Hyphi*	%Hypho**	µH***
KLR	KLLLRLRKLLRR-NH_2	1,577.08	+6	50.0%	50.0%	0.661
KLR^{12}	KLLRRLRKLLRR-NH_2	1,620.11	+7	58.0%	42.0%	0.859
$KLR^{12\text{-}4R}_{12\text{-}4R,7L}$	KLLRRLLKLLRR-NH_2	1,577.08	+6	50.0%	50.0%	0.868

*%Hydrophilicity; **%Hydrophobicity; ***Hydrophobic moment.

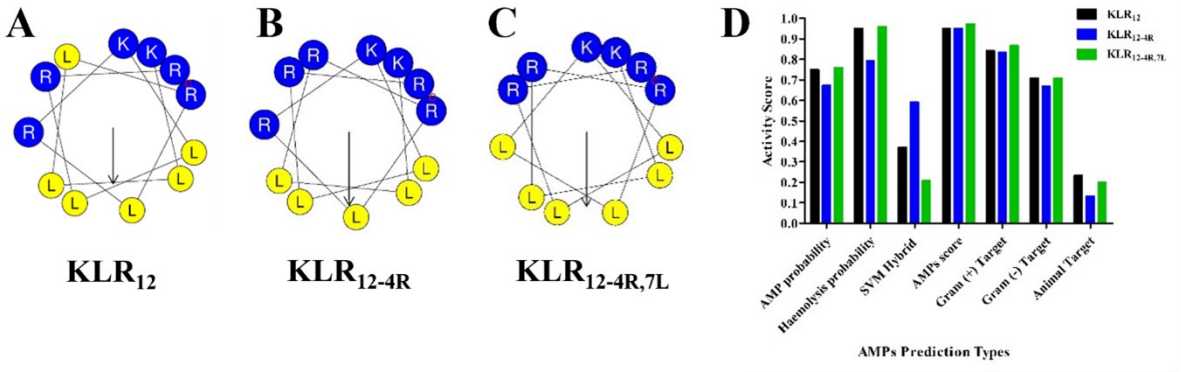
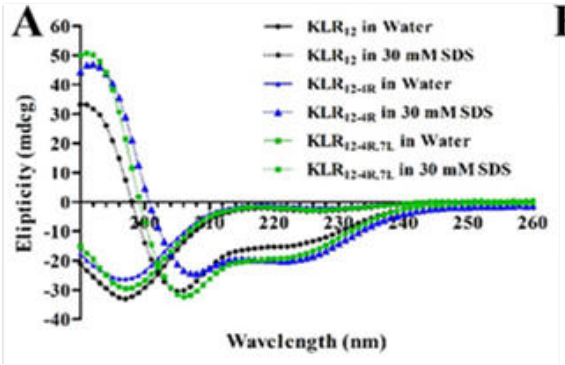


Figure 1. The helical wheel projections of **(A)** native KLR_{12}; **(B)** $KLR_{12\text{-}4R}$; and **(C)** $KLR_{12\text{-}4R,7L}$. Positively charged residues are shown in blue, while hydrophobic residues are shown in yellow. The arrow represents the hydrophobic moment (μH), indicating the overall direction of the amphipathic nature of the α-helix; **(D)** The antimicrobial prediction scores from three prediction tools; MACREL, QSPpred, and AMPfun. The score level: 0, No predicted antimicrobial activity; 0.1 – 0.4, Low antimicrobial activity; 0.5 – 0.7, Moderate antimicrobial activity; 0.8 – 1.0, High antimicrobial activity.



B

%Secondary structure	150 μM peptides in Water			150 μM peptides in 30 mM SDS		
Name	KLR_{12}	$KLR_{12\text{-}4R}$	$KLR_{12\text{-}4R,7L}$	KLR_{12}	$KLR_{12\text{-}4R}$	$KLR_{12\text{-}4R,7L}$
Helix	0	0	0	21.2	31.6	27
Antiparallel	32.6	34.3	33.5	8.5	0	7.9
Parallel	0	0	0	0	0	0
Turn	19.5	18.4	19.3	14.2	15.2	13.1
Random coils	47.9	47.3	47.1	56.1	53.3	52

Figure 2. **(A)** CD spectra of KLR and its derivatives in aqueous solution (solid line) and 30 mM SDS (dotted line) at 25°C. **(B)** Percentage of secondary structure in KLR and its derivatives under different environmental conditions. The data was calculated by BeStSel website. [11]

amphiphilicity, however, maintained the same hydrophilicity and charge as the original structure, as shown in **Table 1 and Figure 1C.** For this reason, the hydrophobic moment of the derivatives increased, which referred to improvement in the amphipathicity of their structure from 0.661 to 0.859 and 0.868, respectively, as shown in **Table 1.**

Prediction tools were used to assess the activity of the modified KLR_{12} derivatives, as depicted in **Figure 1D.** MACREL, was employed, and two types of prediction scores were selected. The AMP Probability indicated that all peptides exhibited moderate antibacterial activity, while the Haemolysis Probability suggested strong haemolytic activity. Additionally, the Quorum Sensing Peptide Prediction (QSPpred) tool was used to evaluate antibacterial properties, with the support vector machine (SVM) hybrid score selected for peptide property prediction. The results suggested that increasing the charge within the peptide structure enhanced its potential to inhibit quorum sensing with moderate activity, whereas peptides with lower charge exhibited reduced quorum sensing activity.

Finally, AMPfun was used to assess four types of prediction scores. The AMP score indicated that all peptides had antibacterial potential, with a particularly strong antimicrobial score against Gram-positive bacteria. In contrast, the peptides exhibited moderate activity against Gram-negative bacteria. Notably, the Animal Target score suggested low interaction with animal cells, indicating a potentially lower cytotoxic effect.

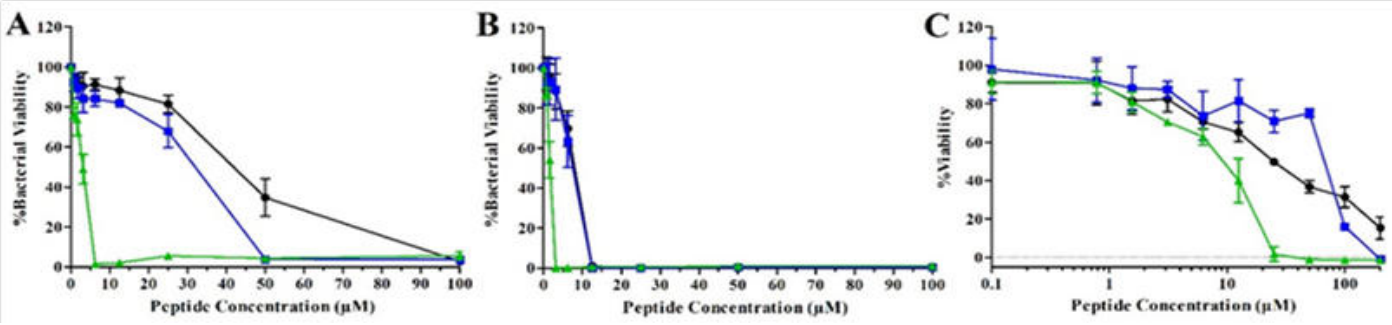


Figure 3. The antimicrobial activity against **(A)** *S. aureus* ATCC 25923; **(B)** *E. coli* ATCC 25922; and **(C)** the cytotoxicity against L929 cell lines. The native peptide, KLR_{12}, is represented by the black line, while $KLR_{12\text{-}4R}$ and $KLR_{12\text{-}4R,7L}$ are shown by the blue and green lines, respectively.

Table 2. MIC, MBC, and IC_{50} values of the native and derivative peptides against candidate Gram-positive, Gram-negative bacteria, and mouse fibroblast L929 cells.

Name	MIC (μM)		MBC (μM)		IC_{50} (μM)
	S. aureus ATCC 25923	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	L929
KLR_{12}	100.0 ± 2.6^a	12.5 ± 0.2^a	> 100	50	24.3 ± 3.5^a
$KLR_{12\text{-}4R}$	50.0 ± 2.3^b	12.5 ± 0.1^a	100	25	48.1 ± 10.5^b
$KLR_{12\text{-}4R,7L}$	6.3 ± 0.9^c	3.1 ± 0.0^b	12.5	6.25	7.2 ± 1.2^c

Statistical differences were calculated using one-way ANOVA followed by Tukey's post hoc test, with $P < 0.05$. Different groups are indicated by letters a, b, and c.

Secondary structure of KLR derivatives in different environments

All of three peptides formed random coil in water as indicated by CD spectra, as depicted in **Figure 2A.** In contrast, in 30 mm SDS all these peptides displayed conformational changes to an alpha helical structure by the presence of a positive ellipticity peak at 190 nm and two minimum ellipticity at 208 nm and 222 nm, as shown in **Figure 2A.** In 30 mM SDS, each peptide underwent a significant conformational change, with helicity increasing from 0 to 21.2%, 31.6%, and 27.0%, respectively, compared with water, as presented in **Figure 2B.** These results indicated that KLR and its derivatives formed an amphiphilic alpha-helical structure in membrane-mimetic environments.

Antibacterial activity against representative Gram-positive and Gram-negative

The effect of charge addition and amino acid rearrangement on the antimicrobial activity of the native KLR and its derivatives was studied using the microbroth dilution method, as illustrated in **Figures 3A and 3B.** The native KLR_{12} demonstrated limited activity against the representative Gram-positive *S. aureus* ATCC 25923 and Gram-negative *E. coli* ATCC 25922, with MIC values of 100.0 ± 2.6 μM and 12.5 ± 0.2 μM, respectively. Meanwhile, the peptide $KLR_{12\text{-}4R}$ with the net charge of +7 showed that the MIC values were moderate against *S. aureus* and *E. coli*, at 50.0 ± 2.3 μM and 12.5 ± 0.1 μM, respectively. Interestingly, the peptide $KLR_{12\text{-}4R,7L}$, which underwent amino acid rearrangement to form amphipathic structure, exhibited strong antibacterial activity, with low MIC values of 6.25 ±0.93 μM and 3.1 ± 0.0 μM against *S. aureus* and *E. coli*, respectively. The MBC values for all peptides were increased compared to MIC and were presented in **Table 2.** These findings suggested that enhancing amphipathicity contributes to improved antibacterial potency, particularly by balancing between hydrophobic and hydrophilic regions in the amphipathic helical structure.

Cytotoxicity of native KLR and its derivative

The L929 mouse fibroblast cell line was used for the cytotoxicity assay against the peptides. The result was displayed in **Figure 3C**, where a concentration ranges from 0.1 to 200 μM of peptides was tested and analysed using the MTT assay to measure mitochondrial activity in living cells. The IC_{50} values of KLR_{12}, $KLR_{12\text{-}4R}$, and $KLR_{12\text{-}4R,7L}$ were 24.3 ± 3.5 μM, 48.1 ±10.45 μM, and 7.2 ±1.2 μM, respectively, as shown in **Table 2**. The results indicate that modifying the sequences through charge addition and rearrangement could influence non-specific toxicity. At MIC values, KLR_{12} and $KLR_{12\text{-}4R}$ exhibited lower toxicity to animal cells, whereas $KLR_{12\text{-}4R,7L}$ demonstrated higher toxicity compared to the others.

Discussion

In this study, modifications of the native KLR_{12} peptide through charge addition and amino acid rearrangement led to improvements in the amphipathic structure of the derivative peptides, increasing their hydrophobic moments. CD spectra indicated that increasing the net positive charge of $KLR_{12\text{-}4R}$ to +7 led to a more complete á-helical formation in a membrane-mimetic environment, reaching 31.6%, compared with $KLR_{12\text{-}4R,7L}$, which possessed lower charges. However, $KLR_{12\text{-}4R,7L}$ exhibited the second-highest level of helicity at 27.0%, suggesting that amphipathic structural arrangement can promote more efficient helix formation compared with KLR_{12}, which exhibited 21.2% helicity. Consequently, these data indicate that amphipathicity can drive greater conformational changes towards helical structures in membrane-like environments, with increased charge playing a crucial role in promoting complete helix formation. Previous studies demonstrated that BotrAMP14 and CortAMP14 similarly formed α-helical structures in an SDS environment, while adopting random coil conformations in aqueous solution. [12]

For the evaluation of antimicrobial potency, a higher helical content in the derivative peptides resulted in greater potency compared with KLR_{12}. The results showed that $KLR_{12\text{-}4R,7L}$ was 16 times more potent against *S. aureus* and 4 times more potent against *E. coli* than the native KLR_{12} in terms of MIC. In contrast, an increase in charge allowed the $KLR_{12\text{-}4R}$ twice as potent against *S. aureus*, while showing no enhanced activity against *E. coli* in terms of MIC. Although $KLR_{12\text{-}4R}$ exhibited a higher helical content based on CD measurements, its hydrophobic content was lower than $KLR_{12\text{-}4R,7L}$. This difference affected the interaction with bacterial cell membranes. Previous studies have indicated that hydrophobicity in helical amphipathic peptides plays a crucial role in penetrating and disrupting the cell membrane. [12] Therefore, $KLR_{12\text{-}4R,7L}$, which possessed greater hydrophobicity, displayed higher potency in bacterial killing.

Interestingly, the relationship between KLR derivatives and L929 cells had slight difference results from bacterial studies. Increasing charge of peptide, $KLR_{12\text{-}4R}$, did not significantly reduce cell viability, with an IC_{50} value of 48.1±10.05 μM compared with KLR_{12}. In contrast, $KLR_{12\text{-}4R,7L}$ caused a greater reduction in cell viability compared with KLR_{12}. This suggests that the increase in charge, which led to a decrease in the hydrophobic content of the peptide structure, helped to minimise non-specific interactions with eukaryotic cells. Conversely, $KLR_{12\text{-}4R,7L}$, which exhibited a higher hydrophobic content, was associated with increased non-specific interactions. Thus, hydrophobicity in the amphipathic helical structure played a vital role in determining specificity towards eukaryotic cells. From the previous studies, the IC_{50} values of two commonly studied antimicrobial peptides, melittin and apamin, were 0.15 μM and 4.2 μM. [13] This indicates that the rational KLR derivatives have lower cytotoxic potency against animal cells, whereas prominent AMPs are considerably more toxic.

Conclusion

The goal of this study was to enhance the antimicrobial potency of KLR through rational design, demonstrating the concept of improved amphipathicity via charge addition and sequence rearrangement. The amphipathic derivatives exhibited greater potency than the native KLR_{12} of which lacks a defined amphipathic structure. Notably, an optimal balance between hydrophobic and hydrophilic regions found in the amphipathic design of $KLR_{12\text{-}4R,7L}$ resulted in the highest potential to combat bacterial pathogens. Peptides stability, efficacy, and pharmacokinetics were not the focus of this study. However, if these factors are addressed through further experimental investigation, both $KLR_{12\text{-}4R}$ and $KLR_{12\text{-}4R,7L}$ could serve as promising candidates for clinical drug development in the treatment of infectious diseases.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data sharing statement

All data analysed during this study were included in the published article. Additional information is available from the corresponding author upon reasonable request for non-commercial purposes.

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