Antibacterial and Antifungal Properties of a Novel Peptide GK-19 and Application on Skin and Soft Tissue Infections by MRSA or Candida Albicans

Chenghua Song[1], RuiChao Wen[1], Jiaxuan Zhou[1], Xiaoyan Zeng[1], Zi Kou[1], Jia Zhang[2], Tao Wang[1], Pengkang Chang[1], Yi Lv[1], and Rongqian Wu[1]

[1]The First Affiliated Hospital of Xi'an Jiaotong University
[2]The Second Affiliated Hospital of Xi'an Jiaotong University

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Abstract

Background and Purpose: The widespread abuse of antibiotics have led to increasing resistance of many important human pathogens. The urgent need to develop novel antimicrobial therapies has stimulated great interest in antimicrobial peptides as therapeutic candidates for the treatment of infectious diseases. Scorpion venom-derived peptide Androctonus Amoreuxi Antimicrobial Peptide 1 (AamAP1) is a new type of host defense peptide with broad-spectrum but moderate antimicrobial property. Most importantly, AamAp1 has been proved to be highly hemolytic and displays significantly high toxicity against mammalian cells. The aim of this study was to evaluate the antimicrobial activity and mechanism of a novel synthetic antimicrobial peptide GK-19 deriving from AamAP1 and its derivatives. Experimental Approach: Five bacteria and three fungi were used to evaluate the antimicrobial effect of GK-19 in vitro. Mouse models of scalded combined with skin and soft tissue infections were used to evaluate the antimicrobial effect of GK-19 in vivo. Key Results: The results indicated that Gk-19 could not only inhibit Gram-positive and Gram-negative bacterial growth but also kill fungi by permeabilizing microbial membrane. Cellular and in vivo studies proved that GK-19 showed negligible toxicity to mammalian cells, low hemolytic activity and high stability in plasma. Furthermore, in mouse models of scald combined with skin and soft tissue infections induced by either Methicillin-Resistant Staphylococcus Aureus (MRSA) or Candida Albicans, GK-19 showed significant antimicrobial and healing effects. Conclusion and Implications: The novel scorpion venom-derived peptide analogue GK-19 is a promising drug candidate in the battle against multi-resistant bacterial and fungal infections.

Title: Antibacterial and Antifungal Properties of a Novel Antimicrobial Peptide GK-19 and Application on Skin and Soft Tissue Infections by*MRSA* or *Candida Albicans*

Short Running Title: A Novel Antibacterial and Antifungal Peptide GK-19 and Its Application

Chenghua Song [1*], *Ruichao Wen* [1], *Jiaxuan Zhou* [1],*Xiaoyan Zeng* [2], *Zi Kou* [1], *Jia Zhang* [4], *Tao Wang* [1], *Pengkang Chang* [1],*Yi Lv* [1, 3], *Rongqian Wu* [1*]

1. National Local Joint Engineering Research Center for Precision Surgery & Regenerative Medicine, Shaanxi Provincial Center for Regenerative Medicine and Surgical Engineering, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, China;

2. Department of Laboratory Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, China;

3. Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China.

4. Department of Gastroenterology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China;

*To whom correspondence should be addressed: Chenghua Song, E-mail: sch871224@xjtu.edu.cn, or Rongqian Wu, E-mail: rwu001@mail.xjtu.edu.cn.

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Author Contribution Statement

Rongqian Wu and Chenghua Song contributed to the design of the study, data analysis and the writing of the manuscript. Ruichao Wen, Jiaxuan Zhou, Zi Kou, Jia Zhang, Tao Wang and Pengkang Chang made substantial contributions to data acquisition. Xiaoyan Zeng and Yi Lv provided technical guidence.

Ethical Approval

All procedures performed in studies involving animals were performed as per the Guidelines for the Care and Use of Research Animals of Xi'an Jiaotong University.

Conflict of interest statement

There is no conflict of interest in submitting this manuscript.

Data Availability Statement

Data of the findings is available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

ABSTRACT

Background and Purpose: The widespread abuse of antibiotics have led to increasing resistance of many important human pathogens. The urgent need to develop novel antimicrobial therapies has stimulated great interest in antimicrobial peptides as therapeutic candidates for the treatment of infectious diseases. Scorpion venom-derived peptide *Androctonus Amoreuxi* Antimicrobial Peptide 1 (AamAP1) is a new type of host defense peptide with broad-spectrum but moderate antimicrobial property. Most importantly, AamAp1 has been proved to be highly hemolytic and displays significantly high toxicity against mammalian cells. The aim of this study was to evaluate the antimicrobial activity and mechanism of a novel synthetic antimicrobial peptide GK-19 deriving from AamAP1 and its derivatives.

Experimental Approach: Five bacteria and three fungi were used to evaluate the antimicrobial effect of GK-19 *in vitro.*Mouse models of scalded combined with skin and soft tissue infections were used to evaluate the antimicrobial effect of GK-19 *in vivo.*

Key Results: The results indicated that Gk-19 could not only inhibit Gram-positive and Gram-negative bacterial growth but also kill fungi by permeabilizing microbial membrane. Cellular and *in vivo*studies proved that GK-19 showed negligible toxicity to mammalian cells, low hemolytic activity and high stability in plasma. Furthermore, in mouse models of scald combined with skin and soft tissue infections induced by either *Methicillin-Resistant Staphylococcus Aureus*(*MRSA*) or *Candida Albicans* , GK-19 showed significant antimicrobial and healing effects.

Conclusion and Implications: The novel scorpion venom-derived peptide analogue GK-19 is a promising drug candidate in the battle against multi-resistant bacterial and fungal infections.

Keywords: antimicrobial peptides; GK-19; skin and soft tissue infections; *MRSA* ;*Candida Albicans* .

Introduction

The misuse and overuse of antimicrobial drugs in clinic is very common in recent years. The drug resistance of the top five pathogenic bacteria in nosocomial infection continues to deteriorate (Hofer, 2022; Larsson & Flach, 2021). Some drug-resistant bacteria and even superbugs are no longer rare in clinic (Ghosh, Sarkar, Issa & Haldar, 2019; Song et al., 2020). Moreover, the research cost of new antibiotic is relatively high and the development cycle is much longer than the breeding speed of drug-resistant microbes. The number of new antibiotics approved every year is declining (Hofer, 2019). Therefore, in response to the rising challenge of drug resistance, it is particularly urgent to look for alternative antibiotics that won't induce drug-resistance (Ardal et al., 2020; Bell & MacLean, 2018).

Antimicrobial peptides (AMPs), a kind of active peptides produced by biological immune system, is an important class of host-defense factor (Hancock, Alford & Haney, 2021). Compared with traditional antibiotics, it has been proved that antimicrobial peptides not only have broad-spectrum antibacterial property, but also have significant effects on anti-fungi, immune regulation, anti-viruses, anti-parasite and even inhibition of tumor cells (Cebrian, Xu, Xia, Wu & Kuipers, 2021; Nibbering et al., 2019; van der Weide et al., 2019). More importantly, most AMPs are thought to kill bacteria cells by a mechanism of inducing membrane damage with subsequent leakage of bacterial cellular content/debris, which won't induce drug resistance, making them attractive scaffolds for the creation of therapeutics superior to traditional antibiotics (Brogden, 2005; Mwangi et al., 2019; Pambos & Kapanidis, 2019). Thus, it is expected to be a powerful weapon for infections, especially the resistance of micro-organisms infection.

The venoms of scorpions, which have been proved to kill gram-negative and gram-positive bacteria, fungi, viruses and even tumor cells, are a rich source of antimicrobial peptides. *Androctonus Amoreuxi*Antimicrobial Peptide 1 (AamAP1) (Almaaytah, Zhou, Wang, Chen, Walker & Shaw, 2012), identified from the venom derived cDNA library of the North African scorpion *Androctonus Amoeruxi* , is a new type of host defense peptide. With broad-spectrum antimicrobial property, it has been prospected that AamAP1 kills bacteria cells through membrane disruption, which won't induce bacterial resistance. However, there are still some disadvantages in using AamAP1. It displays moderate activity against gram-positive and gram-negative bacteria. Except that, AamAP1 and its derivatives (A3 and AamAP1-Lysine) (Almaaytah, Abualhaijaa & Alqudah, 2019; Almaaytah, Farajallah, Abualhaijaa & Al-Balas, 2018; Almaaytah et al., 2014) are unstable in biological body and have predicted short half-life of less than 2 hours. In addition, natural AamAP1 is difficult to obtain and its purity is very low. More importantly, it is proved to be highly hemolytic and displays significantly high toxicity against mammalian cells. All these disadvantages have greatly limited its clinical application prospects.

In this study, the amino acid sequence of a new scorpion venom derived from antimicrobial peptide analogue GK-19 was designed. The antimicrobial activity and mechanism of GK-19 for both bacteria and fungi were investigated. At the same time, the therapeutic effect of GK-19 on mouse models of scald combined with skin and soft tissue infections (SSTIs) by *Methicillin-Resistant Staphylococcus Aureus*(*MRSA*) or*Candida Albicans* (*C. Albicans*) was studied to determine its applicability. The results demonstrated that GK-19 has broad-spectrum antimicrobial activities for both bacteria and fungi, negligible toxicity and excellent healing effect for SSTIs induced by either*MRSA* or *C. Albicans in vivo* .

Methods

Characterization of the Physicochemical Properties of the Antimicrobial Peptides.

The physicochemical properties of the designed antimicrobial peptides were calculated using the peptide property calculator tools including SOPMA secondary structure prediction method, Expasy ProtParm tool and INNOVAGEN PROTEOMICS tools based on the peptide sequence. Thermal shift assay was conducted on the BIO-RAD CFX Connect^{TM} Real-Time system and data analysis was processed using the CFX Maestro software.

Antimicrobial Peptides Stability Assay.

Antimicrobial peptides were dissolved in water or rat serum respectively with the concentration of 10 mg/mL. The solution was incubated at 37 °C, 200 rpm for different times and diluted 50 times. High performance liquid chromatography (HPLC) was conducted on a Waters HPLC system, which consisted of a 2996 photodiode array detector, a 2695 separation module and a temperature control column oven.

Column: Sinochrom ODS-BP, 5 μm, 4.6 * 250 mm, Dalian Elite Analytical Instruments Co., Ltd. China.

Mobile phase: A: 100% ACN + 0.1% TFA; B: 100% Water + 0.1% TFA.

Gradient elution: 0 - 20 min, 95% B - 100% A; 20 - 25 min, 100% A.

Detection wavelength: 220 nm.

Flow rate: 1 mL/min.

Sample volume: 50 μL (0.2 mg/mL).

Bacteria and fungi Strains Preparation and Growth Conditions.

Pseudomonas Aeruginosa (*P. Aeruginosa* , ATCC27853),*Methicillin-Resistant Staphylococcus Aureus* (*MRSA* , 2104270609, clinical isolated strain), *Escherichia Coli* (*E. Coli* , ATCC25922), *Klebsiella Pneumoniae* (*K. Pneumoniae* , ATCC700603), *Enterococcus Faecalis* (*E. Faecalis* , ATCC29212), *Candida Krusei* (*C. Krusei* , ATCC6258),*Candida Albicans* (*C. Albicans* , ATCC90028) and*Candida Glabrata* (C. *Glabrata* , ATCCMYA-2950) were obtained from the First Affiliated Hospital of Xi'An Jiaotong University. Bacterial strains were cultured in MHA or MHB. Fungal strains were cultured in PDA or PDB. Their concentrations were determined by the absorbance of 600 nm: 1 $OD_{600\ nm} = 4.8 \times 10^8$ CFU/mL

Antimicrobial Assay.

Strains were incubated in MHB or PDB respectively. After growing for 12-16 hours at 37 °C, 200 rpm, 100 μL of 1×10^6 CFU/mL of the strains were seeded into 96 well plates and incubated with fresh culture medium containing various antimicrobial peptides (peptide concentration: 0, 0.5, 1.0, 2.0, 3.0, 5.0, 10, 15 and 20 μM) for 12 hours. Absorbance at 600 nm was measured by using the Multilabel Reader VARIOSKAN FLASH (Thermo Fisher). Microbial viability were calculated through this formula: Viability (%) = (mean absorbance value of treatment group – blank) / (mean absorbance value of control group - blank) × 100.

AntimicrobialMechanism.

Scanning electron microscopy (SEM, GeminiSEM 500, Carl Zeiss) was used to investigate the antimicrobial mechanism of the designed peptides. Different strains were incubated with 50 μM of GK-19 for 2 h at 37 °C, 200 rpm. The solution was centrifuged at 4000 rpm for 10 min and washed with PBS for twice. Supernatant was removed and 2.5% of glutaraldehyde solution was added for fixation of the microbial clumps. After a standard SEM sample preparation process, samples were observed by SEM.

Cell Culture.

Rat pulmonary artery smooth muscle cells (RPASMCs, primary cells separated by our laboratory) and Human keratinocytes cells (HaCaT cells, MINGJING BIOLOGY, Shanghai) were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVECs, BeNa Culture Collection, China) were cultured in MCDB131 medium supplemented with 20% FBS and growth factors additives. Human embryonic kidney cells (293, Procell Life Science & Technology Co., Ltd., Wuhan) were cultured in minimum Eagle's medium (MEM) supplemented with 10% FBS. All cells were cultured at 37 °C, 5% CO2.

In Vitro **Cytotoxicity Assay.**

Cells were inoculated in 96 well plates at 5×10^3cells/well and cultured at 37 °C under 5 % CO_2. Supernatant was removed, cells were washed three times with PBS. 100 μL of fresh culture medium containing different antimicrobial peptides were added respectively (peptide concentration: 0, 1.0, 2.0, 5.0, 10, 25 50 and 100

μM). 10 μL of CCK-8 reagent was added 48 hours later, followed by another 2 hours' incubation. Absorbance at 450 nm was measured using the same Multilabel Reader. Cellular growth viability was calculated through this:

Viability (%) = (mean absorbance value of treatment group / mean absorbance value of control group) × 100.

In Vivo **Cytotoxicity Assay.**

Male Kunming mice weighing ~ 25 g were supplied by the Medical Animal Test Center of Xi'an Jiaotong University and all the experiments were performed as per the Guidelines for the Care and Use of Research Animals. 100 μL of GK-19 (5 μmol/kg or 25 μmol/kg) was injected intravenously (n = 5, per group). Mice weight was recorded daily. Mice blood samples were collected from eyeballs for biochemical analysis. Major organs including lung, heart, kidney, liver and spleen were harvested for hematoxylin-eosin staining on 1, 3 and 7 days after injection.

Hemolysis Assay.

Hemolysis assay was carried out as reported in the literature (Lei et al., 2018) and the concentration of GK-19 were 5, 25, 50, 100, 200 and 400 μM. The percentage of hemolysis was calculated using this formula: Hemolysis (%) = [($OD_{576\ nm}$ of the peptide solution - $OD_{576\ nm}$ of the negative control) / ($OD_{576\ nm}$ of the positive control - $OD_{576\ nm}$ of the negative control)] × 100. The tests were repeated for six times, and the data were expressed by the mean and standard deviation of six replicates.

TheMouse Models of Scald Combined withSSTIs.

Mouse models of scald combined with SSTIs by *MRSA* or*Candida Albicans* were constructed (Bjorn et al., 2015). Normal Kunming mice weighing ~ 25 g were anesthetized by isoflurane. After skin preparation and disinfection, a custom-made circular iron (diameter: 8 mm) (Shown in Fig. S4) was applied to both sides of the spine of the mice for 10 s at 300 °C, resulting in four scalded wounds. Two days after scalding, skin tissues of the scalded area were removed carefully, about 30 μL of 1×10^7CFU/mL of *MRSA* or *Candida Albicans* were applied evenly onto the surface of the wound. Mice were put back to the original feeding environment after the microbial fluid was completely absorbed.

In Vivo **Anti-SSTIs Assay.**

Two days after microbe inoculating, scalded wound of each mouse was divided into four groups randomly (n = 10). Saline solution, antimicrobial peptide, clindamycin hydrochloride (for*MRSA*) or fluconazole (for *C. Albicans*) was applied evenly onto the surface of the wound for twice (morning and evening) per day and the drug concentration was 50 μM. Mice were put back to the original feeding environment after the microbial fluid was completely absorbed. The treatment lasted for 12-16 days. Photographs of the wound area were taken every two days and wound area were measured using the Image J software. Skin tissues of the wound area were removed carefully at different time points and weighted in a sterile environment. For *MRSA* infection, 10 mg of the wound tissues were dispersed into 1000 μL of sterile saline solution and the tissues solution were grounded into suspension in a tissuelyser (Tissuelyser LT, Servicebio). Then, the tissue suspension was diluted with sterile saline solution for 10 times and plated on MHA with a sample volume of 50 μL. For *Candida Albicans* infection, 10 mg of the wound tissues were dispersed into 200 μL of sterile saline solution and the tissues solution were also grounded into suspension in the tissuelyser. The tissue suspension was then plated on PDA directly with a sample volume of 20 μL. After culturing overnight, the colonies were counted using the Image J software. In addition, skin tissues of wound area were removed carefully at different time points and dispersed into 4% paraformaldehyde solution for further use. The immunohistochemistry analysis of hematoxylin-eosin staining was carried out by Servicebio (Wuhan, China).

Statistical Analysis.

Data were expressed as means ± standard deviation. The statistical difference between two groups was determined using a one-way ANOVA with the following p- values: * $p < 0.05$, ** $p< 0.01$, *** $p < 0.001$.

Materials.

All the antimicrobial peptides were purchased from Shanghai GL Biochem Ltd. and their purity were determined to be higher than 98% using reversed-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry. Mueller-Hinton Broth (MHB), Mueller-Hinton Agar (MHA), Potato Dextrose Broth (PDB), Potato Dextrose Agar (PDA), clindamycin hydrochloride and fluconazole were purchased from Solarbio Life Science (China). CCK-8 kit was obtained from Dojindo Co. LTD (Japan). Urea assay kit, alanine aminotransferase assay kit, aspartate aminotransferase assay kit and creatinine assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (China). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Thermo Fisher. All the reagents were of commercial special grade and were used without further purification.

Results

Peptide Functional Screening Shown a Prolonged Half-Life of GK-19.

Based on the amino acid sequence of AamAP1, its derivatives A3 and AamAP1-Lysine, a glycine residue was introduced to the N-terminal of AamAP1-Lysine. As shown in Tab. S1, with the introduction of glycine, the hydrophobicity of GK-19 decreased slightly and the helicity increased. More importantly, the predicted half-life was greatly extended, suggesting that its antimicrobial activity may also be enhanced. The melt curve and melt peak in Fig. S1 showed that the changes of amino acid sequence and numbers had no effect on the thermal stability of peptides. Then, the stability of AamAP1 and GK-19 in water and rat serum was investigated by HPLC. When preserved in water, both GK-19 and AamAP1 retained their structural stability for 48 hours or more, even at physiological temperature of 37 °C (Fig. S2(a)). However, the structure of AamAP1 changed and the position of its chromatographic peak moved backward when incubated in rat serum for only two hours. More than 65% of AamAP1 peptides were degraded after seven hours of co-incubation with rat serum. In contrast, GK-19 maintained its stability even after 24 hours of co-incubation with rat serum and more than 50% of GK-19 still remained (Fig. S2(b, c)). These results encourage us to study the antimicrobial activity of GK-19.

GK-19 Potently Kills a Broad Range of both Bacteria and Fungi by Permeabilizing the Microbial Membrane.

As shown in Fig. 1(a) and Tab. S2, GK-19 exhibited potent antibacterial activity against three Gram-positive and two Gram-negative bacteria with much lower minimal inhibitory concentration (MIC) values ranging between 3 to 10 μM (*E. Coli* (3 μM), *K. Pneumoniae* (5 μM),*P. Aeruginosa* (5 μM), *E. Faecalis* (3 μM) and*MRSA* (5 μM)) in comparison to that of AamAP1 (> 20 μM). In contrast, Aamap1 had almost no bactericidal effect within the same concentration range. Additionally, except for *MRSA* , GK-19 had stronger antimicrobial activity against the other four common clinical pathogenic bacteria than AamAP1-Lysine. To assess the possible mechanism of GK-19 action on bacteria, two Gram-positive bacteria (*E. Faecalis* and *MRSA*) and two Gram-negative bacteria (*E. Coli* and *K. Pneumoniae*) were incubated with GK-19 at a concentration of 50 μM for 2 hours. As illustrated in Fig. 1(b), GK-19 led to the formation of blebs (roughness) and irregularly shaped holes on the bacterial membrane of both *E. Faecalis* and *MRSA*(Gram-positive bacteria), whereas untreated controlled groups revealed a significant difference in membrane morphology with smooth and complete bacterial membrane. For Gram-negative bacteria *E. Coli* and *K. Pneumoniae* , after treatment with GK-19, irregular abysses, pores and rupture were also observed obviously. Considering the known mechanisms of the antibacterial peptides, it is conceivable that disruption of the bacterial membrane and/or cell wall structure is the cause of lethality in bacteria treatment with GK-19.

With the control of bacterial infections, fungal infection is becoming an important disease gradually (Fisher et al., 2012). Studies have shown that the prevalence of superficial mycosis worldwide is as high as 20% ˜ 25%, indicating that fungi have partially replaced bacteria as the main pathogen (Brown, Denning & Levitz, 2012). What's more, over-treatment of bacteria can also lead to the growth of fungi. Untreated aggravation of deep SSTIs can cause rapid necrosis of tissues and organs, or it can also spread into the blood, resulting in fungal septicemia, sepsis, and the patient's life will be threaten (Brown, Denning, Gow, Levitz, Netea

& White, 2012; Echaiz, Burnham & Bailey, 2013). Therefore, the situation of infections caused by fungi is increasingly serious. Encouraged by the excellent antibacterial activity of GK-19, we then used three clinical common *candida* to investigate the antifungal activity of GK-19. As shown in Fig. 2(a) and Tab. S2, GK-19 also exhibited distinguished antifungal activity with much lower minimal inhibitory concentration (MIC) values ranging between 5 to 10 μM (*C. Krusei* (5 μM), *C. Albicans* (10 μM) and *C. Glabrata* (10 μM)) in comparison to that of AamAP1. AamAp1 could only inhibit 90% of*C. Krusei* and showed almost no bactericidal effect on *C. Albicans* and *C. Glabrata* within the same concentration range. In addition, AamApl-Lysine showed similar antifungal activity as GK-19. These results suggested that the antimicrobial activity of GK-19 against these microorganisms was maintained or even enhanced by introducing a glycine residue to N-terminal of AamAP1-Lysine. Meanwhile, we also studied the antifungal mechanism of GK-19 by using the same method as bacteria. As illustrated in Fig. 2(b), after treatment with GK-19, the cells of *C. Krusei* also showed membrane disruption and pore formation in most cells in comparison with untreated group. Deep cracks and rupture appeared on the membranes of *C. Albicans* and *C. Glabrata,* slightly different from the membrane morphology of *C. Krusei* . It was conceivable that the disruption of the fungal membrane and/or cell wall structure was also the cause of lethality in fungi treatment with GK-19. Collectively, these data suggested that GK-19 exhibited potent and quick antibacterial and antifungal activity by permeabilizing the microbial membrane, indicating that it might be used for the treatment of infections both *in vitro* and *in vivo*without inducing resistance.

GK-19 Showed Negligible Toxicity both *in Vitro* and*in Vivo*.

The main obstacle for the clinical use as promising therapeutics of antimicrobial peptides is their side effects, such as cytotoxicity and hemolysis etc. To better understand the toxicity of the GK-19, several normal mammalian cells, including rat pulmonary artery smooth muscle cells (RPASMCs), human keratinocytes cells (HaCaT), human umbilical vein endothelial cells (HUVECs) and human embryonic kidney cells (293), were chosen to evaluate the cytotoxicity induced by GK-19. As shown in Fig. 3(a), almost no apoptotic cell was observed in the presence of GK-19 at effective antimicrobial concentration (10 μM). At 10 times of the effective antimicrobial concentration (100 μM), treatment with GK-19 resulted in a cell viability of less than 20% for RPASMCs, HUVECs and 293 cells. However, more than 42% of the HaCaT cells were still survived after treated with 100 μM of GK-19. In addition, we evaluated the hemolytic activity of GK-19 *in vitro* . As shown in Fig. 3(b), GK-19 exhibited negligible hemolytic activity even at relatively high concentrations (100 μM). This result was in stark contrast to the findings obtained with AamAp1, which caused almost 100% hemolysis at the same concentration (Almaaytah, Zhou, Wang, Chen, Walker & Shaw, 2012). To further evaluate the safety of GK-19 *in vivo,* mice were injected intravenously with GK-19 at dosage of 5 μmol/kg or 25 μmol/kg. As shown in Fig. 3(c), the slices of mice major organs treated with 25 μmol/kg of GK-19 indicated that no observable tissue damage, necrosis or inflammation was observed, either at the short term of 1 day or the long term of seven days. Meanwhile, the results of liver and renal function test showed no difference with that in healthy mice (Fig. S3(a, b, c, d)). We also monitored the changes in body weight of mice for seven days. As shown in Fig. S3(e), mice remained healthy as indicated by the gradual gain in body weight, which further demonstrated that the GK-19 was relatively safe for use *in vivo* . Collectively, these data confirmed a favorable toxicity/safety profile of GK-19 *in vitro* and *in vivo* .

GK-19 Promoted the Wound Healing of Mice against SSTIs Caused by *MRSA*.

Encouraged by the promising *in vitro* and *in vivo* results obtained above, we further assessed the *in vivo* antimicrobial efficacy of GK-19 using a mouse model of scalded combined with SSTIs by*MRSA* . Fig. 4(a, b) showed the gross appearances of the skin wounds and quantitative analysis of wounds area. After scald, the skin of the scalded area became slightly focal yellow and hard immediately. The wounds were treated with SS, AamAp1, CDHC or GK-19 respectively two days later and observed until they were healed. On day 2, the skin wounds of mice were dark brown and edematous. Scabs were tightly adherent to the wound surface on day 4 with GK-19 or CDHC, whereas the skin tissue edema was aggravated and some pus were observed in AamAp1 and SS treated groups. On day eight, the size of the scabs treated by GK-19 or CDHC were significantly reduced with more than 45% of the wounds area healed in comparison with that treated by

AamAp1 (˜ 27%) or SS (˜ 13%). On day 12, the scab tissues of GK-19 or CDHC treated wounds fell off and wound areas were greatly reduced. In contrast, scabs still tightly adhered to the tissue treated by AamAp1 or SS. However, the wound healing rate of AamAP1 treated group (˜ 77%) was significantly faster than that of SS treated group (˜ 54%), indicating that AamAP1 also had certain ability to promote wound healing. The wounds in both GK-19 and CDHC treated groups had completely disappeared on day 16, whereas ˜ 6% or ˜ 27% of unhealed wounds remained observable in AamAp1 or SS treated groups. These results suggested that Gk-19 was comparable to CDHC and more effective than AamAP1 in promoting wound healing of SSTIs caused by *MRSA* . Meanwhile, the colonized *MRSA* in skin and subcutaneous tissues were quantified. As shown in Fig. 4(c, d), compared with SS groups, treatment with GK-19 led to a significant reduction of *MRSA* , with more than 95% of *MRSA* being suppressed at day eight, which was comparable to the inhibition rate of CDHC (˜ 95%). However, AamAP1 groups also showed *MRSA* inhibition rate of more than 90%, but a much lower healing speed. This could be attributed to the serious inflammatory reaction caused by the existed abundant*MRSA* at the early period of infection. To further evaluate the antibacterial and healing effect of GK-19 on infected wounds, histological analysis was performed. As shown in Fig. 6(a), compared with normal skin tissues, ubiquitous inflammatory cell infiltration (mainly neutrophil), large amounts of fragments of pus cells, necrotic tissues and *MRSA* clumps were observed in the skin tissues two days after infected by *MRSA* . On day 16, the healing effect of GK-19 was comparable to that of CDHC, with completely healed wound tissues. No *MRSA* clumps and pus cell fragments were observed, and inflammatory cells were distributed less or not observed. What's more, a large number of fibroblasts, neovascularization and even newborn hair follicle tissues were observed in the subcutaneous tissue just below the wound surface of GK-19 treated groups, indicating the good healing. In contrast, massive neutrophil infiltration and a lot of smaller*MRSA* clumps were still observed in SS treated groups. In addition, the skin tissues of AamAP1 treated groups were nearly healed, but very fewer *MRSA* clumps could also be observed. Correcting, these data suggested that GK-19 could penetrate through the skin into the infected sites to eradicate *MRSA* , indicating its potential as part of a class of antibiotics with significant therapeutic potential for external use.

GK-19 Promoted the Wound Healing of Mice against SSTIs Caused by *Candida Albicans*.

Serious SSTIs are likely to develop into systemic infections that threaten the patient's life and health (Pfaller & Diekema, 2007). Considering the growing threat of fungal infections and the excellent*in vitro* inhibitory effect on *Candida* of GK-19, a mouse model of scalded combined with SSTIs by *C. Albicans* was constructed just like mentioned above. The wounds were treated for twice every day with saline solution (SS), AamAp1, fluconazole (FCZ) or GK-19 respectively and observed until they were healed. Similar results as in antibacterial studies were observed in Fig. 5(a, b). On day 10, the size of the scabs treated by GK-19 or FCZ were significantly reduced with more than 95% of the wounds area healed in comparison with that treated by AamAP1 (˜ 88%) or SS (˜ 75%). The wounds in both groups treated by GK-19 and FCZ had completely disappeared on day 12, whereas ˜ 5% or ˜ 14% of unhealed wounds remained observable in AamAp1 or SS treated groups. These results suggested that GK-19 was comparable to FCZ and more effective than AamAP1 in aspect of promoting wound healing of SSTIs caused by *C. Albicans* . Meanwhile, we also quantified the colonized *C. Albicans* in the infected skin and soft tissues. As shown in Fig. 5(c, d), the fungal load in the tissues infected by *C. Albicans* was much lower, which would be the reason of the faster healing speed of SSTIs induced by *C. Albicans* . Compared with SS treated groups, treatment with GK-19 could led to a significant reduction in the colonization of *C. Albicans* in subcutaneous tissues, with more than 97% of *C. Albicans* being suppressed at day eight, which was comparable to the inhibition rate of FCZ (˜ 98%). In contrast, there were still more than 20% residual *C. Albicans* in group treated by AamAP1 in comparison with SS treated group. On day 12, there were no *C. Albicans* detected in groups treated by GK-19 or FCZ, whereas a few residual *C. Albicans* were still observed in SS and AamAP1 treated groups. These results suggested that GK-19 indeed promoted wounds healing by inhibiting fungal proliferation. What's more, histological analysis was also performed. As expected, the number of fungal clumps observed in the tissues infected by *C. Albicans* were smaller and the clumps tended to be round or oval, which was corresponded to the previous results of fungal load (Fig. 6(b)). Ubiquitous inflammatory cell infiltration, monocyte, lymphocytes and neutrophils were infiltrated and necrotic tissues were also observed in the skin tissues two

days after infected by*C. Albicans* . On day 12, the wounds of both GK-19 and FCZ treated groups were completely healed, with no observable *C. Albicans*clumps and inflammatory cells. What's more, the skin structure was almost completely intact with a large number of neovascularization and newborn hair follicle tissues, indicating the comparable healing effect of GK-19 to FCZ. In contrast, there were still massive neutrophil infiltration, lymphocytes, a small amount of necrotic tissues and a lot of smaller *C. Albicans* clumps being observed in SS treated groups and wounds tissues were still scabby. In addition, the skin tissues of AamAP1 treated groups were nearly healed, but a small number of very small *C. Albicans* clumps could also be observed. Correcting, these data suggested that GK-19 could also penetrate through the skin into the infected sites to eradicate *C. Albicans* , indicating its potential as part of a class of antibiotics with significant therapeutic potential for SSTIs caused by both bacteria and fungi.

Discussion

As the cornerstones of modern public health, antibiotics have made remarkable contributions to anti-infection. However, the widespread use and even abuse of antibiotics have led to increasing resistance of many important human pathogens (Curren et al., 2022). There is an urgent need to find new antibiotics that won't induce resistance. Unlike antibiotics, which inhibit microbial growth by blocking synthesis of microbial macromolecules in various ways, such as inhibiting protein, cell wall and nucleic acid synthesis, antimicrobial peptides are generally considered to kill microbes by forming pores in microbial cell membranes and causing leakage of intracellular substances (Brogden, 2005). In this paper, a novel 19-residue antimicrobial peptide called GK-19 is characterized. Synthetic GK-19 showed a wide range of antibacterial and antifungal activities. In particular, our study showed that the GK-19 can indeed induce the formation of pores by interacting with the cell membranes of bacteria and fungi, suggesting that GK-19 may not induce drug resistance. However, the interaction between GK-19 and the microbial cell membrane appears to be the first step, their cationic nature may also interfere with polyanionic intracellular components such as DNA, RNA and ribosomes etc. Therefore, further studies will be needed to determine the exact antimicrobial mechanism of GK-19.

GK-19 showed significant antimicrobial and healing effect on scald combined with SSTIs both by *MRSA* and by *C. Albicans*.However, this is just a preliminary application study and the method of administration was simple transdermal delivery. It was not investigated whether GK-19 could retain its superior antimicrobial activity after being injected into the mice body by tail vein, or whether it could be used for deep tissue infections, such as systemic infection, lung infection and other treatments (Curren et al., 2022). In this paper, we had proved that GK-19 showed low toxicity to mammalian cells, low hemolytic activity and high stability in plasma, which provided a theoretical basis for administration of GK-19 through the tail vein. In addition, the administration of daily and continuous dosing was used for SSTIs treated with GK-19 and this administration was quite cumbersome. Therefore, whether GK-19 can be combined with other technologies, such as hydrogels, to prepare antimicrobial skin dressing (Cao, Wu, Cheng, He, Chen & Zhou, 2021; Liang, He & Guo, 2021; Liang, Li, Huang, Yu & Guo, 2021), from which peptide drugs can be released slowly for a long time, so as to reduce the frequency of administration and provide maximum convenience for patients, warrants further investigation. At the same time, the main reason affecting the application of existing antimicrobial peptides in deep tissue infections is that the antimicrobial peptides will be cleared soon after entering the blood circulation system, resulting in the presence concentration in the lesion area is lower than the effective concentration. The increase of the concentration of drug administration will enhance the toxic and side effects to the body. For that reason, we are also considering that whether we can try to combine GK-19 with nanotechnology (Gao & Zhang, 2021; Lei et al., 2018; Makabenta, Nabawy, Li, Schmidt-Malan, Patel & Rotello, 2021). We expect to find resolutions to package GK-19 into nanomaterials for delivery, or directly modify GK-19 with nanomaterials. We expect that this combination will preserve the superior antibacterial properties of GK-19, enhancing its residence time in the bloodstream, increasing the binding time and presence concentration in the lesion area and increasing the antimicrobial effect with lower administration dosage.

In conclusion, our results demonstrated the potentials of GK-19, a designed antimicrobial peptide analogue

deriving from Scorpion Venom, as a possible antimicrobial drug candidate which could not only inhibit Gram-positive and Gram-negative bacterial growth but also kill fungi by permeabilizing microbial membrane. GK-19 showed low toxicity to mammalian cells, low hemolytic activity, high stability in plasma and significant antimicrobial and healing effects on scald combined with SSTIs induced by either *MRSA* or *C. Albicans in vivo.* Thus, the novel scorpion venom-derived peptide analogue GK-19 is a promising drug candidate in the battle against multi-resistant bacterial and fungal infections.

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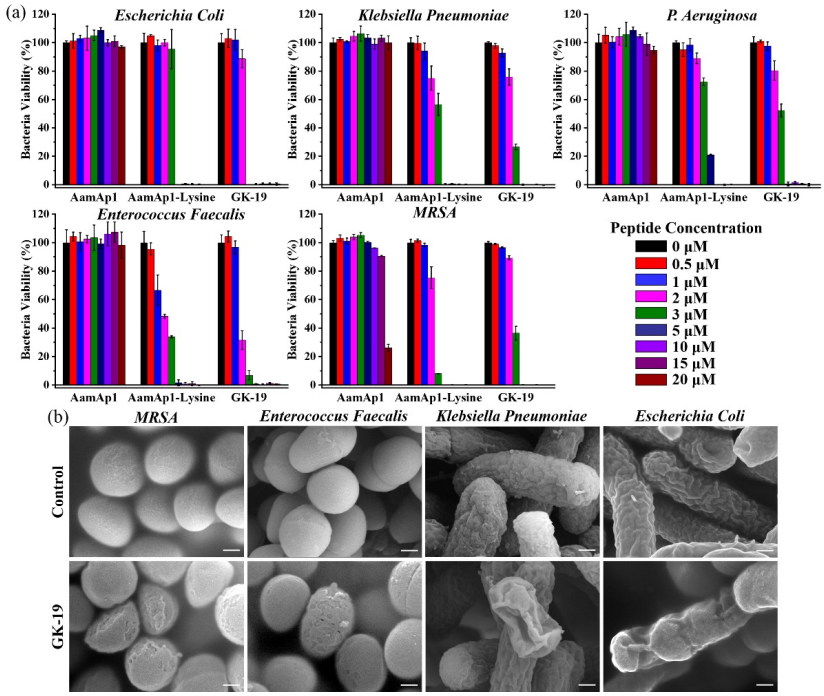


Fig. 1. Antibacterial activity of AamAP1, AamAP1-Lys and GK-19. (a) Dose-dependent survival of*Escherichia Coli* , *Klebsiella Pneumoniae* , *P. Aeruginosa* , *Enterococcus Faecalis* and*Methicillin-Resistant Staphylococcus Aureus* (*MRSA*) treated with different antimicrobial peptides for 12 h. Bacteria (1×10^6 CFU/mL) were incubated with the peptides at concentrations varying from 0 to 20 μM. (n = 5) (b) SEM images of different bacteria 2 hours after incubated with GK-19 at a concentration of 50 μM. Scale bar: 200 nm.

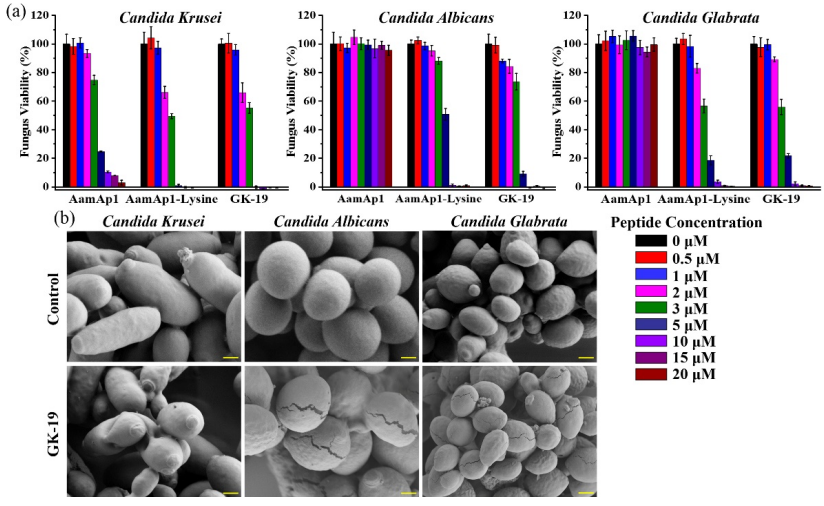


Fig. 2. Antifungal activity of AamAP1, AamAP1-Lys and GK-19. (a) Dose-dependent survival of *Candida Krusei, Candida Albicans* and *Candida Glabrata* treated with different antimicrobial peptides for 12 h. Fungi (1×10^6CFU/mL) were incubated with the peptides at concentrations varying from 0 to 20 μM. (n = 5) (b)

SEM images of different fungi 2 hours after incubated with GK-19 at a concentration of 50 μM. Scale bar: 1 μm.

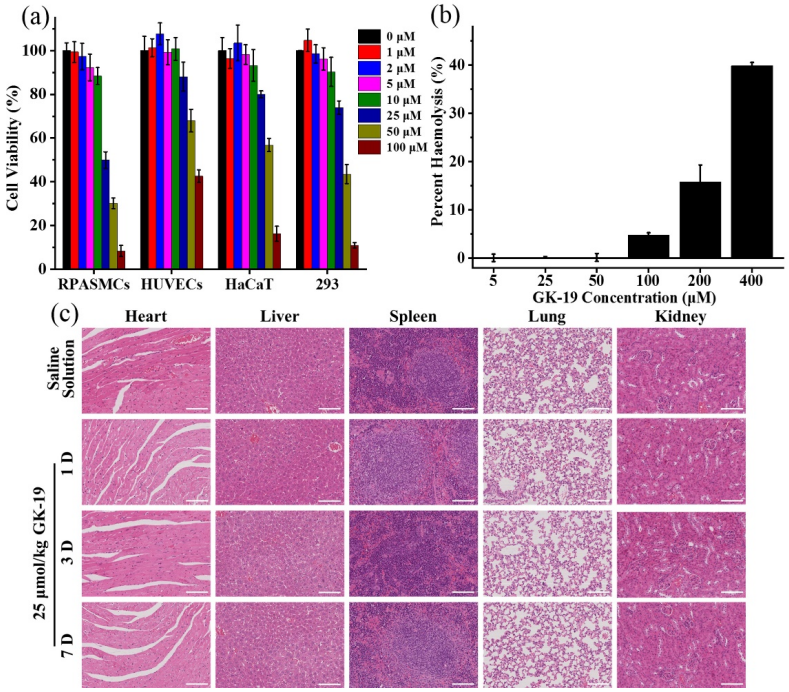


Fig. 3. (a) Toxicity of GK-19 to mammalian cells. RPASMCs, HUVECs, HaCaT and 293 cells were cultured in medium containing different concentrations of GK-19 (0 -100 μM) for 48 hours. (n = 5). GK-19 causes a negligible toxicity to mammalian cells at effective antimicrobial concentrations. (b) Hemolytic activity of GK-19 on rat erythrocytes (c) Histology analysis of various organs of the mice treated with saline solution or GK-19 (25 μmol/kg), respectively. Heart, liver, spleen, lung and kidney were harvested at day 1, 3 and 7. No observable tissue damage, necrosis and inflammation were observed. Scale bar: 200 μm.

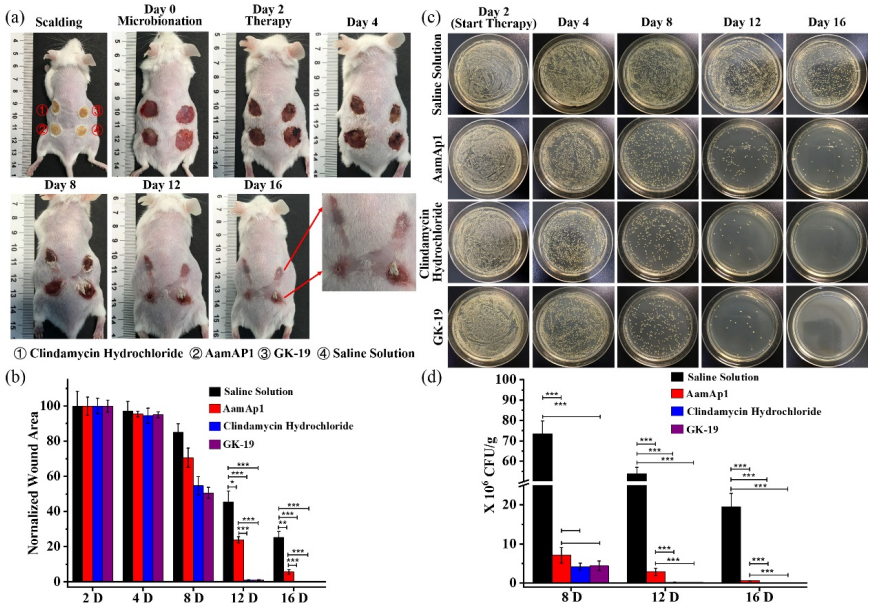


Fig. 4. Bactericidal and healing effect of GK-19 on *MRSA* inoculated onto mouse scalded dorsal skin. (n = 10) (a) Time course images of mice infected by*MRSA* . Infected dorsal skin of mice was treated with saline solution, AamAp1, clindamycin hydrochloride or GK-19 respectively for twice every day. The concentrations of drugs were 50 μM. (b) Quantitative analysis of wounds areas using image J software. (c, d) Time courses bacterial count in *MRSA* infected wounds after treated with different antimicrobial peptides. Skin tissues of different groups were sampled at day 2, 4, 8, 12 and 16. Tissue suspensions were diluted with sterile saline solution and 50 μL of tissue suspensions were then plated in MHA culture plates. Images were taken 18-24 hours after inoculation. The quantitative analysis of colonies was counted using Image J software or visual observation.

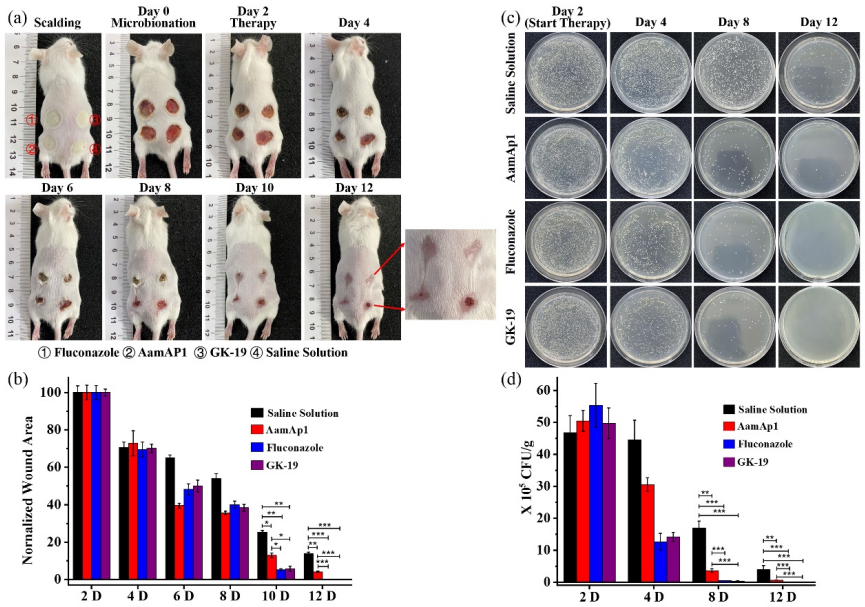


Fig. 5. Fungicidal and healing effect of GK-19 on *Candida Albicans* inoculated onto mouse scalded dorsal skin. (n = 10) (a) Time course images of mice infected by *Candida Albicans* . Infected dorsal skin of mice was treated with saline solution, AamAp1, fluconazole and GK-19 respectively for twice every day. The concentrations of drugs were 50 μM. (b) Quantitative analysis of wounds areas using image J software. (c, d) Time courses fungal count in *Candida Albicans* infected wounds after treated with different antimicrobial peptides. Skin tissues of different groups were sampled at day 2, 4, 6, 8, 10 and 12. Tissue suspensions were diluted with sterile saline solution and 20 μL of tissue suspensions were then plated in PDA culture plates. Images were taken 18-24 hours after inoculation. The quantitative analysis of colonies was counted using Image J software or visual observation.

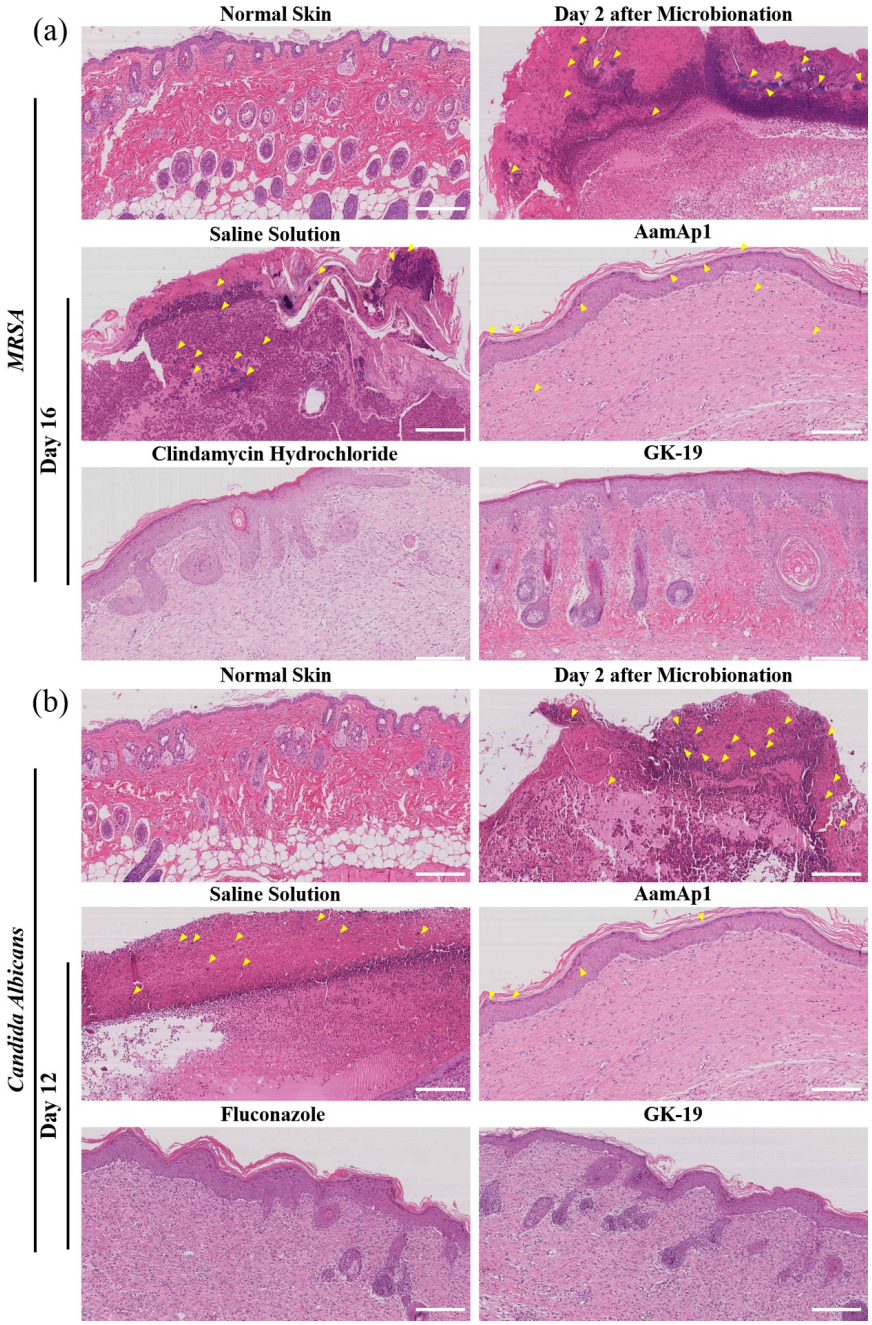
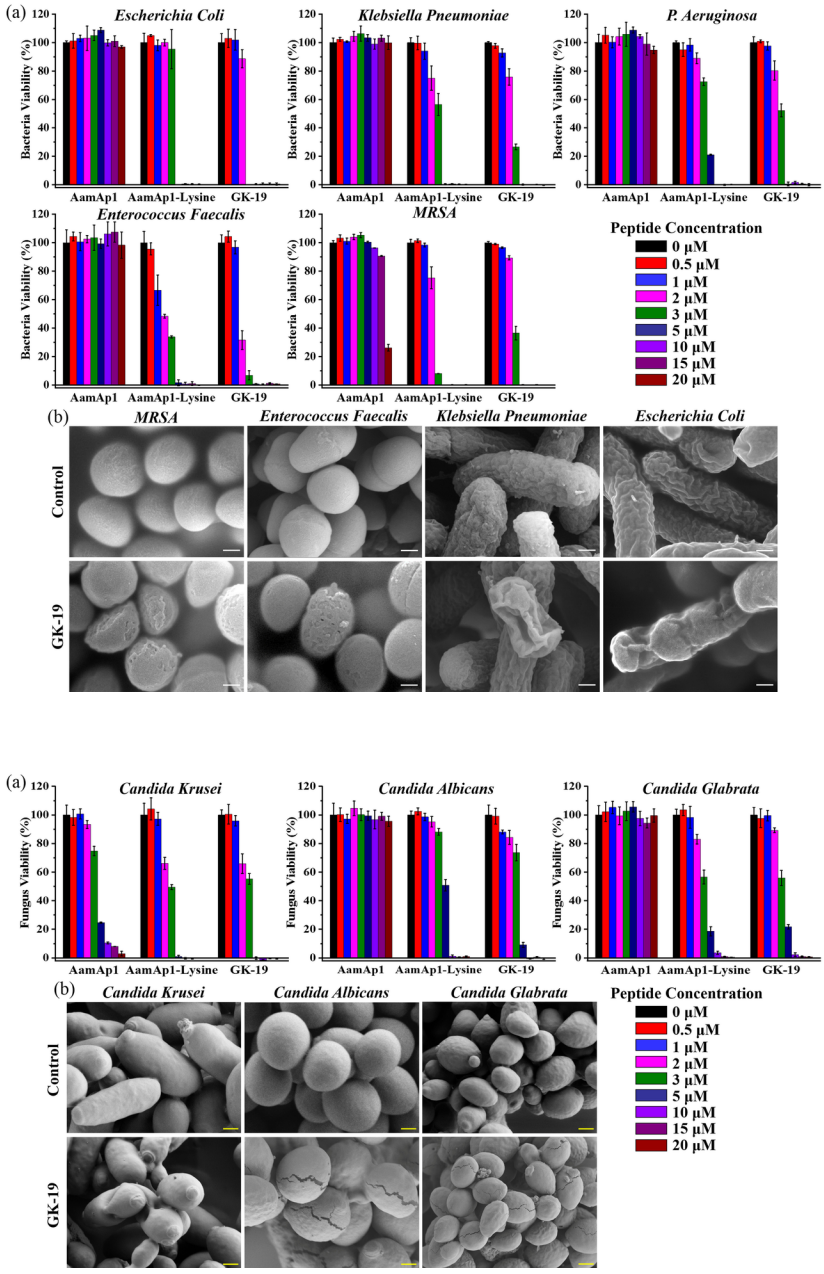
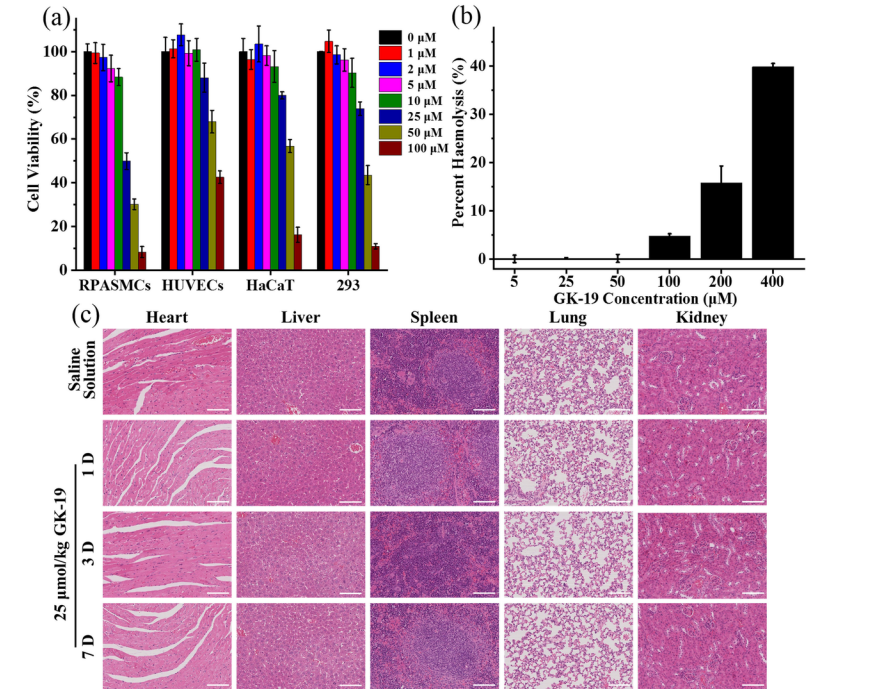
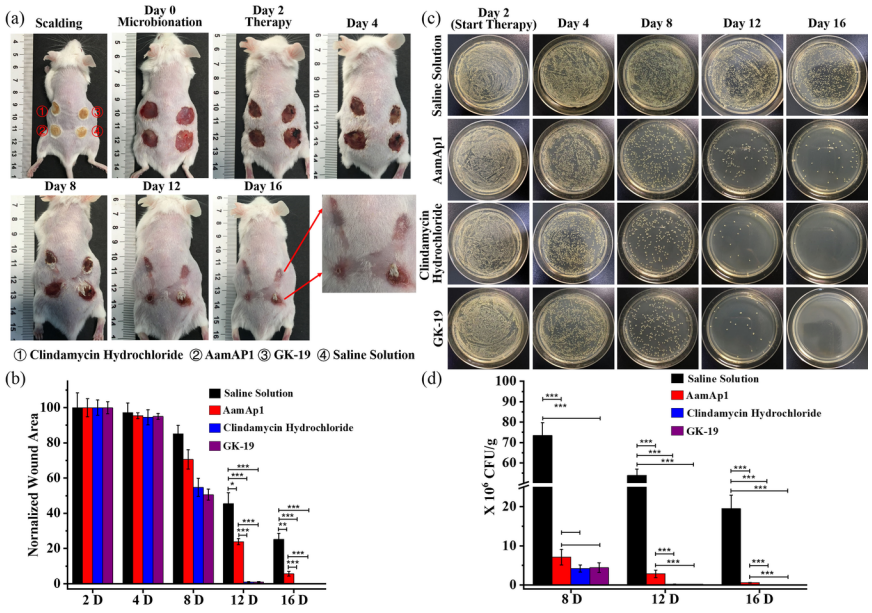
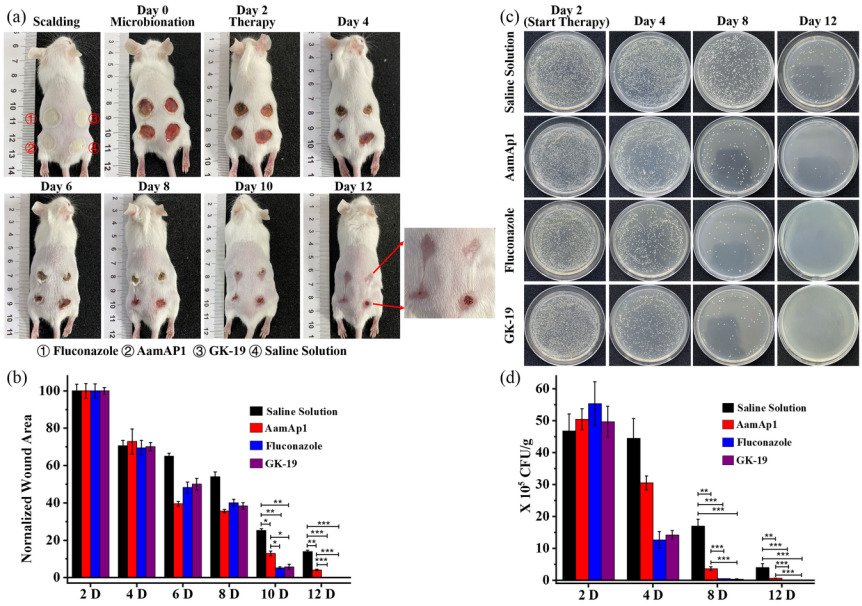


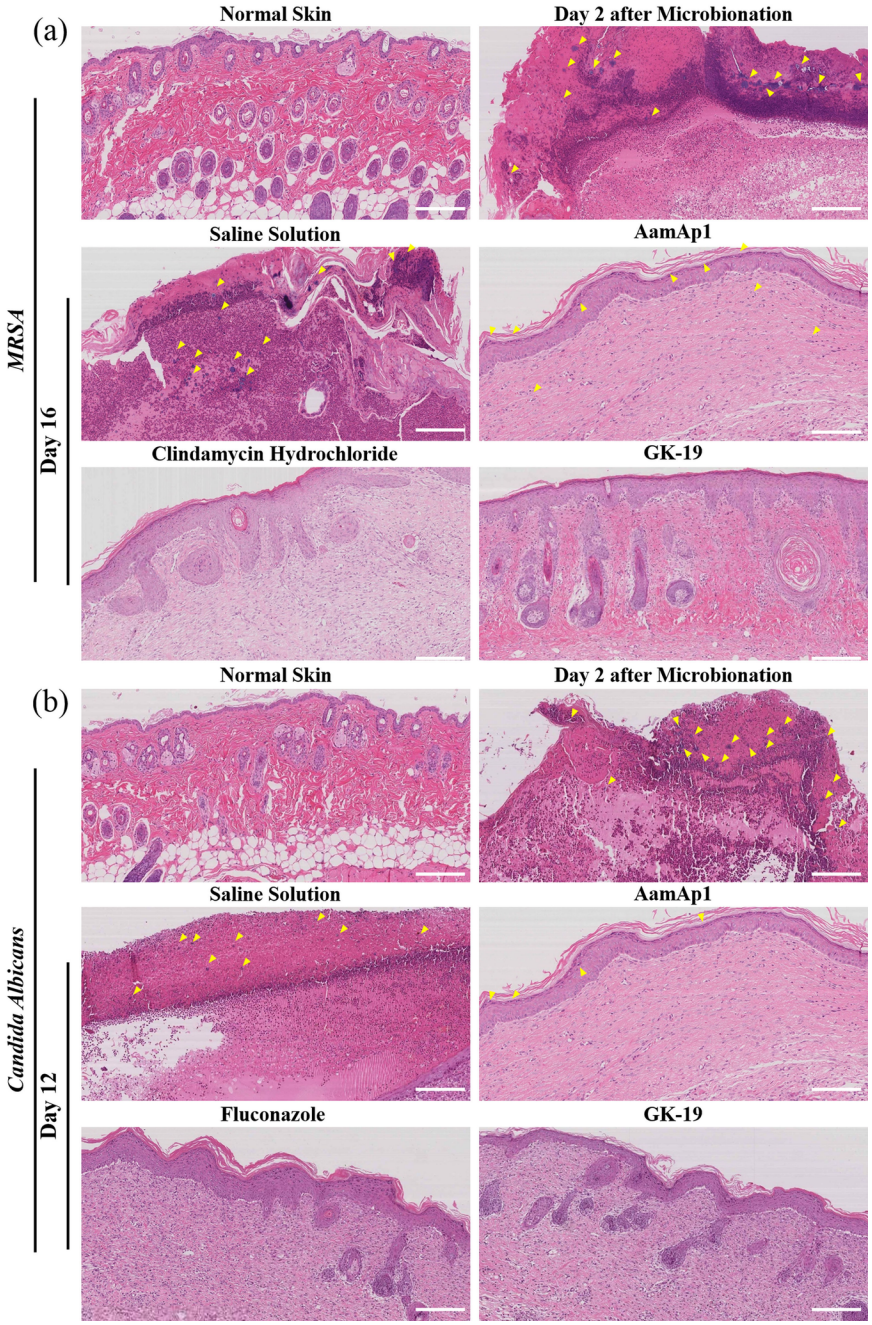
Fig. 6. Histology analysis of skin samples of the scalded mice infected by *MRSA* (a) *or Candida Albicans* (b). The skin wound was treated with saline solution, AamAP1, GK-19, clindamycin hydrochloride (for *MRSA*infection) or fluconazole (for *Candida Albicans* infection), respectively and the concentrations of drugs were 50 μM. Skin samples were harvested at different time points. Scale bar: 200 μm.











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