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RIP-V improves murine survival in a sepsis model by down-regulating RNAIII expression and α-hemolysin release of methicillin-resistant *Staphylococcus aureus*

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Received August 16, 2014, accepted September 19, 2014

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Pharmazie 70: 81–87 (2015) *doi: 10.1691/ph.2015.4750*

Staphylococcus aureus is associated with serious invasive infections and high mortality rates due to a large number of toxins released. The persistent increasing resistance of *S. aureus* has driven the need for new anti-infection agents and innovative therapeutic strategies. RNAIII-inhibiting peptide (RIP) has been reported to reduce bacterial virulence by interfering with *S. aureus* quorum sensing system. The present study aimed to investigate whether two new RIP derivatives (RIP-V and RIP-L) could improve the survival rate of mice in a MRSA sepsis model. We found that neither anti-bacterial nor cell toxicity were displayed by all RIPs *in vitro*. *In vivo* protective effects were observed using a MRSA-induced mice sepsis model. Among RIPs, RIP-V exhibited the strongest protection function on mice survival and inhibition of pathological damages. Our studies firstly verified that RIPs could inhibited the RNAIII expression of *S. aurues* isolated from liver tissue of BALB/c mice. Moreover, RIP-V exhibited the strongest inhibitory effect on RNAIII and can decrease markedly the secretion of α-hemolysin in liver. These findings indicate that RIP-V might be considered as a potential and specific drug candidate for treating *S. aureus* infections, especially for MRSA.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of bloodstream infection (Chen et al. 2012). It is associated with severe invasive infections (McDougal et al. 2010) and high mortality rates (Rodvold and McConeghy 2014). Vancomycin has been a last-resort antibiotic against MRSA for many years. However, persistent MRSA therapeutic failures have been reported under vancomycin (Sakoulas and Moellering 2008; Holmes et al. 2011). Moreover, a clinical outbreak of linezolid-resistant *S. aureus* was reported in an intensive care unit. The rapid development of bacterial resistance to multiple antibiotics makes it necessary to immediately change the currently employed antibacterial strategies.

Quorum sensing (QS) exists widely in bacteria and plays a role as a cell–cell communication process (Novick and Geisinger 2008). Interfering with the bacterial QS system can significantly reduce bacterial virulence and control the formation of bacterial biofilms while not killing the bacteria directly. This leads to pathogenic bacteria experiencing a lower selective pressure on survival, and reduces the development of drug resistance (Ng and Bassler 2009; Rasko and Sperandio 2010). Hence, targeting QS systems is a more promising strategy than traditional approaches in antibacterial research. RNAIII-inhibiting peptide (RIP), as a QS inhibitor, could inhibit the RNAIII-activating protein (RAP)/target of RAP (TRAP) system in staphylococci (Balaban et al. 2001).

RNAIII-inhibiting peptide (RIP) has attracted a lot of attention and its roles have been recognized in improving the survival rate of *S. aureus*-infected mice (Giacometti et al. 2005; Ghisellii et al. 2006). The RIP derivative YSPWTNF reduced lethality in combination with cefazolin, vancomycin, imipenem or antimicrobial peptide (BMAP-28) in mouse models of staphylococcal sepsis (Giacometti et al. 2005, Ghiselli et al. 2006). Moreover, YKPITNF exhibited a higher inhibitory effect than YSPWTNF in *S. aureus*-induced cellulitis model (Gov et al. 2001). In order to further enhance the activity of YKPITNF *in vivo*, isoleucine residues at its position 4 was replaced by valine or leucine. These three amino acids are all hydrophobic amino acids and their structure is similar.

Here, the therapeutic efficacy of RIP YKPWTNF(RIP-I) and its derivatives YKPVTNF (RIP-V) and YKPLTNF (RIP-L) were evaluated in MRSA-induced mice sepsis (Table). Antibacterial activity and cell toxicity were assayed by minimal inhibitory concentration (MIC), growth curve and MTT determination *in vitro*. Mice survival rates, histological changes, and bacterial load in blood, lung and liver tissues were assessed in murine sepsis model. The RNAIII mRNA expression and α-hemolysin secretion of MRSA was analyzed in a murine sepsis model.

2. Investigations and results

2.1. RIPs had no antibacterial effect in vitro

RIPs did not kill *S. aureus* ATCC29213 or MRSA *in vitro* (MIC > 256 μg/ml) (Fig. 1A). For *S. aureus* ATCC29213, oxacillin and vancomycin exhibited MICs of 0.25 and 0.5 (μg/ml, respectively. In contrast, antibiotics exerted weak

Table: Peptides investigated.

Designation	Amino acid sequence	Molecular weight	Purity (%)
RIP-V	YKPVTNF$_{CONH2}$	867	97.36
RIP-L	YKPLTNF$_{CONH2}$	881	98.13
RIP-I	YKPITNF$_{CONH2}$	881	97.19
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ$_{CONH2}$	2846	96.32

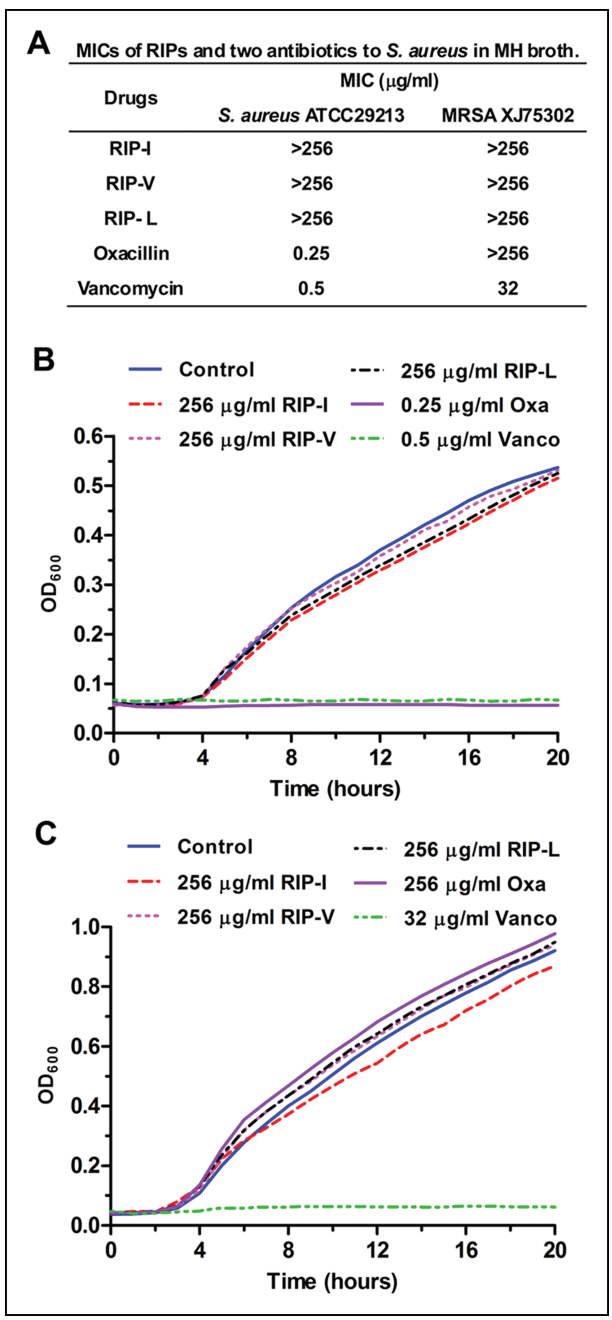

Fig. 1: *In vitro* antibacterial activities of RIPs. (A) MICs of RIPs, oxacillin and vancomycin. *S. aureus* ATCC29213 or MRSA XJ75302 was grown overnight in nutrient broth. Mueller–Hinton (MH) broth (100 μl) containing bacteria (5×10^5 CFU /ml) was added to 100 μl of the culture medium containing the test compound (0.12 μg/ml to 256 μg/ml in serial twofold dilutions). The plates were incubated at 37 °C for 20 h. (B, C) Time-kill curves of RIPs. RIP-I, RIP-V, RIP-L, oxacillin and vancomycin were added to cell cultures containing *S. aureus* ATCC29213 (B) or MRSA XJ75302 (C) to a final concentration 256 μg/ml. The optical density of the cell suspensions was measured automatically at 600 nm with the automated Bioscreen C system in regular intervals of 1 h for 20 h.

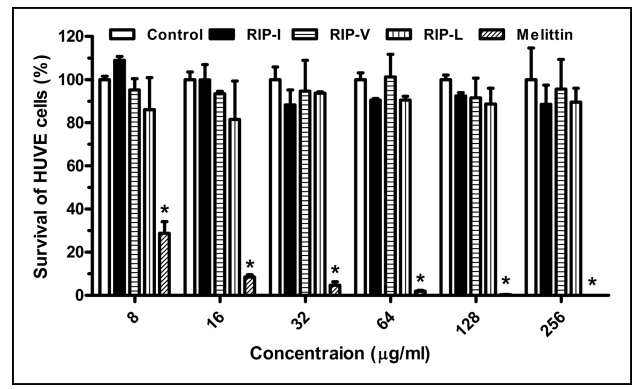

Fig. 2: Toxicity of RIPs *in vitro*. The absorbance at 490 nm of HUVE cells after incubation without or with various concentrations of RIPs or melittin for 48 h. Each plot was obtained from a representative experiment, and the data points are the means of four replicates ± standard deviation. The data were shown as mean ± SD values of 4 samples. *$P < 0.001$ *vs.* control group.

effects against MRSA, with MIC values of > 256 and 32 (μg/ml for oxacillin and vancomycin, respectively. Similarly, the results of growth curves also showed all RIPs did not inhibit the growth of *S. aureus* ATCC29213 or MRSA (Fig. 1B and C).

2.2. *In vitro toxicity*

To ensure the safety of RIPs, we investigated their cytotoxicity to human umbilical vein endothelial (HUVE) cells *in vitro*. As shown in Fig. 2, there was no significant difference on cell viability with increasing RIPs concentration ($P > 0.05$). But melittin, an antimicrobial peptide, exhibits powerful lytic activity against eukaryotic cells only at 8 μg/ml. These results imply that RIPs have a relatively wide safety range for potential clinical applications.

2.3. *RIP therapy in vivo*

Peritoneal infection with MRSA resulted in mice death within 4 days. The i.p. administration of any of the RIPs (RIP-I, RIP-V, or RIP-L) at 10 mg/kg significantly improved the animal survival rate to 18.18%, 45.45%, 27.27%, respectively, compared with 0% in the model group and 27.27% in the 5-mg/kg-vancomycin group (Fig. 3A). The mortality rate in the infected group without treatment was 100% at 4 days after infection (Fig. 3A). Survival was associated with reductions in the bacterial titers in the blood of mice inoculated with MRSA, from 2.64×10^6 CFU/ml in the infected group to 1.45×10^5, 7.64×10^3, 5.17×10^4, and 5.56×10^4 CFU/ml in the RIP-I, RIP-V, RIP-L and vancomycin treatment group, respectively (Fig. 3B). Compared with the model group, RIP-V decreased significantly the bacterial CFU in the blood of BALB/c mice (Fig. 3B).

2.4. *Morphology of lung and liver tissue samples*

The normal alveolar structure disappeared in the model group of MRSA-infected mice (Fig. 4A), with congestion, hemorrhage,

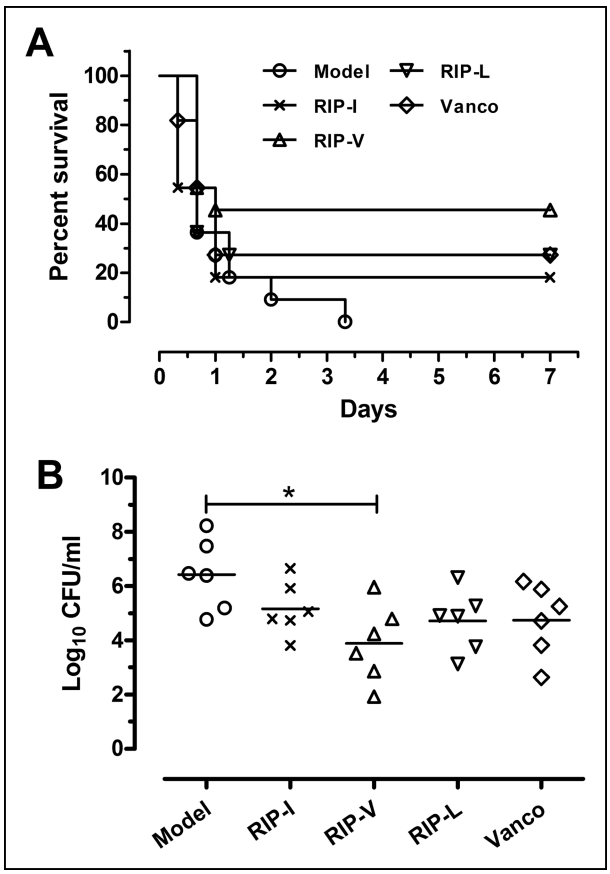

Fig. 3: RIPs rescued MRSA-infected BALB/c mice from sepsis. (A) Survival of BALB/c mice (n = 11 mice per group) inoculated with MRSA and treated with 10 mg/kg RIP-V, 10 mg/kg RIP-L, 10 mg/kg RIP-I, 5 mg/kg vancomycin, or same amount of sterile water by i.p. injection at 1 h after infection. (B) Colonization of MRSA inoculum in the blood cultures of BALB/c mice after RIP-treated or vancomycin-treated for 8 h (CFUs/ml of blood sample). $*P < 0.05$ *vs*. model group.

necrosis, and liquefaction being observed and the infiltration and accumulation of a large number of neutrophils. Some neutrophils infiltrated the local alveolar space and alveolar septum, and there was partial alveolar fusion in the vancomycin-, RIP-L-, and RIP-I-treated groups (Fig. 4A). The morphological structure of lung tissue was very similar between the RIP-V-treated group and the normal control group. The normal alveolar structure was evident with little alveoli integration and only slight thickening of the alveolar wall (Fig. 4A). Swelling of liver cells, and hepatic sinusoidal dilation and congestion were observed in the model group (Fig. 5A). All of these phenomena were less evident in the vancomycin- and RIP-treated groups (Fig. 5A). RIP-V exerted the strongest protective effects on the liver of MRSA-infected mice.

The organ protective effects were associated with reductions of the bacterial titers in the liver and lung tissues of mice inoculated with MRSA (Fig. 4B and 5B). RIP-V decreased obviously about 3 log units of CFU in the tissue of liver or lung in mice with lethal MRSA infection.

2.5. RIPs can obviously down-regulate the expression of RNAIII in MRSA-infected BALB/c mice

In order to determine the relative expression of *RNAIII*, MRSA were isolated for total RNA extraction from part of the lung or liver in the BALB/c mice treated by RIPs. Real time RT-PCR was used to determine the expression levels of *RNAIII* and *16S rRNA* in lung and liver tissue of mice infected by MRSA after RIPs therapy. The expression of *RNAIII* was lowered markedly in all RIPs-treated groups than that in the model group ($P < 0.05$). There were both obvious differences between the RIP-V- and RIP-I-treated groups in lung and liver tissue ($P < 0.05$) (Fig. 4C and 5C).

2.6. RIPs can effectively inhibit α-hemolysin secretion from S. aureus in infected BALB/c mice

Compared to the model group, RIP-V was obviously effective at inhibiting the secretion of hemolysins in MRSA-infected mice (Fig. 6A). In addition, there were significant differences between RIP-V and vancomycin treatment group ($P < 0.05$) (Fig. 6A). Western blot results showed that relative to the model group, the protein levels of α-hemolysin were clearly decreased in all RIPs-treated groups in the liver of MRSA-infected mice ($P < 0.01$), especially in the RIP-V-treated group ($P < 0.001$) (Fig. 6B).

3. Discussion

RAP induces the phosphorylation of TRAP and in contrast, RIP inhibits this process. The sequence of native RIP was identified by Prof. Balaban as YSPXTNF, where X could be a Cys, a Trp, or a modified amino acid (Balaban et al. 1998). According to the sequence similarity between RIP (YSPXTNF) and N-terminal 4-9 amino acid residues of *S. aureus* RAP (YKPITN), RAP and RIP might compete on binding to the same site on the receptor (Balaban et al. 1998). Also, it was considered to enhance the inhibitory activity by increasing the sequence similarity of RIP to the amino terminal of RAP (Gov et al. 2001). They designed a series of RIP derivatives by simulating the amino terminal sequence of RAP peptides. Surprisingly, the RIP derivative YKPITNF was the most effective inhibitor of cellulites *in vivo* (Gov et al. 2001). In addition to the last amino acids, the sequence of RIP derivative (YKPITNF) and RAP N-terminal sequence (YKPITN) are exactly identical.

As we know, pharmacological activity of peptides is closely related to their spatial structure. Therefore, novel and efficient RIP derivatives may be produced by such a method, which the original amino acid was replaced by amino acids with similar structure and propertyies. Valine, leucine and isoleucine all belong to hydrophobic amino acids, and their structure is very similar. Consequently, two new RIP derivatives (RIP-V and RIP-L) were produced by replacing isoleucine with valine and leucine. Our results showed RIP-V was more effective than RIP-I in the murine *S. aureus* sepsis model, which demonstrated that the design method of amino acid substitution was feasible. Similar to RIP-I, RIP-V and RIP-L both display neither bactericidal nor bacteriostatic activities *in vitro*. However, it is worth noting that all RIPs (256 μg/ml) are devoid of detectable cytotoxicity in normal mice HUVE cells. In sharp contrast to RIPs, melittin, an antibacterial peptide owning strong bactericidal activity (Raghuraman and Chattopadhyay 2007), shows serious cell toxicity to HUVE cells. In the light of our current results, none of the three RIPs could play direct antibacterial effect *in vitro*, and it could be inferred that RIPs might be used safely for treatment of *S. aureus* infections *in vivo*.

Using a severe sepsis model based on the lethal quantity injection of *S. aureus* to mice, RIP-V administrated groups markedly decreased disease severity in mice. Results *in vivo* showed that all of the synthetic RIPs were effective in suppressing *S. aureus*-induced sepsis and improving the survival rate of mice. RIP-V exhibited stronger protection activities than RIP-I and RIP-L on MRSA infections. A particularly exciting finding was that 10 mg/kg RIP-V had a higher efficacy than 5 mg/kg vancomycin against MRSA infection. Furthermore, histological experiments showed that a single dose of RIP-V could effectively suppress the bacterial titers in the blood of *S. aureus*-infected mice and

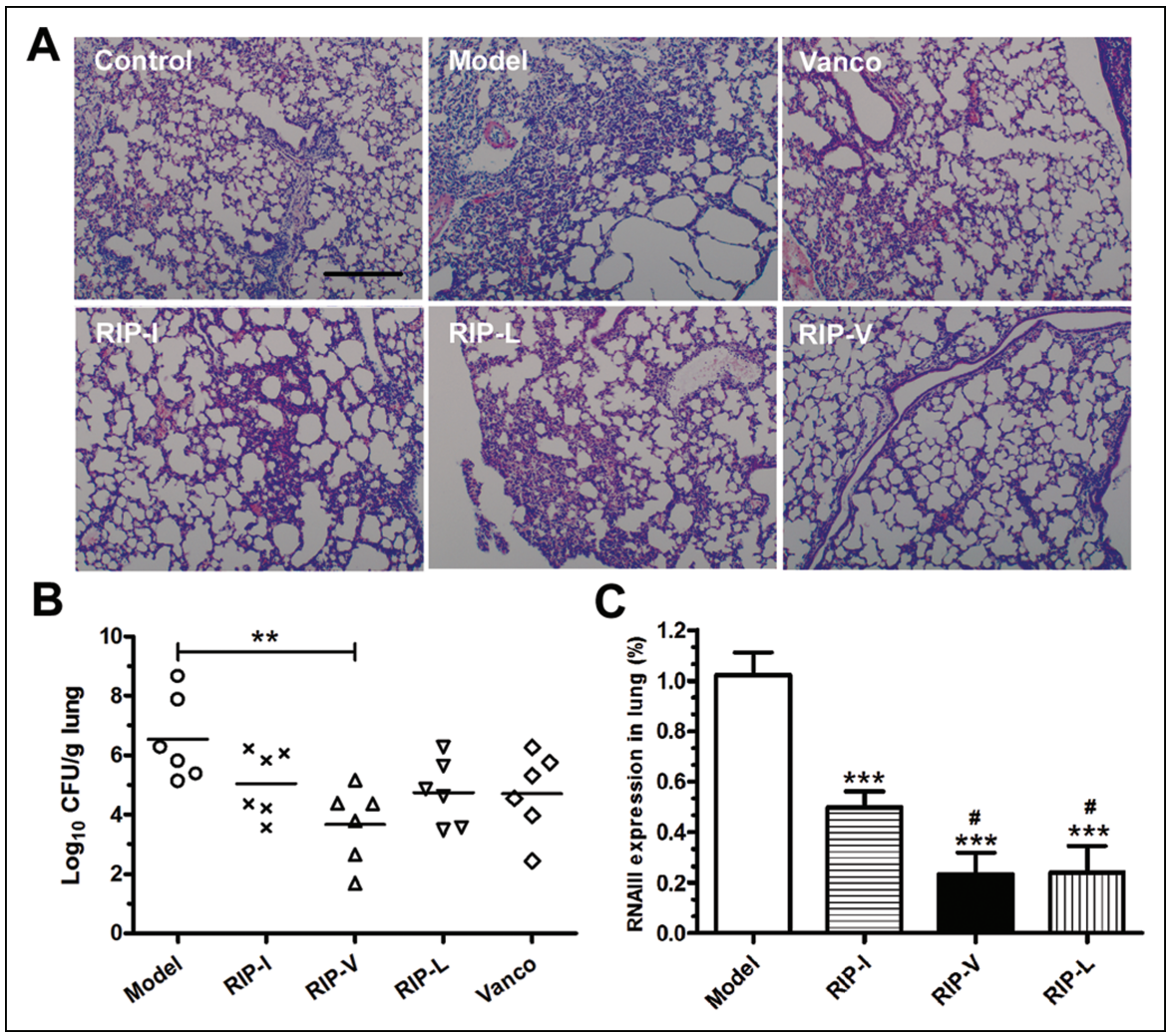

Fig. 4: Protective effect of RIPs on lung in MRSA-infected mice by inhibiting the expression of RNAIII mRNA. BALB/c mice were infected for 1 h and then were administered a single i.p. dose of 10 mg/kg RIP-V, RIP-L, RIP-I or 5 mg/kg vancomycin to evaluate the morphology change in lung. (A) Morphology of the lung was show with H.E. staining in BALB/c mice infected by MRSA. The original magnification is 100 × , and the bar length represents 200 μm. (B) Colonization of MRSA inoculum in the lung tissue of BALB/c mice after RIP-treated or vancomycin-treated for 8 h (CFUs/g of lung sample). $^{**}P < 0.01$ *vs*. model group. (C) Quantitative RT-PCR analysis of RNAIII mRNA level in MRSA isolated from infected mice lung tissues following treatment with 10 mg/kg different RIPs. RNA expression was quantified after comparison with an internal gene (*16S rRNA*). Data are expressed as the mean ± SD ($n = 3$). $^{***}P < 0.001$ *vs*. model group; $^{\#}P < 0.05$ *vs*. RIP-I group.

prevent pathological changes associated with extensive lung and liver damage in the model mice.

S. aureus is not only responsible for many infections, but also a frequent cause of sepsis in humans, a disease associated with high incidence, mortality and cost. The armamentarium of virulence factors produced by *S. aureus* is extensive, including α-hemolysin, sortase, and panton-valentine leukocidin that are implicated in the pathogenesis of severe infections. Of these toxins, α-hemolysin has proved to be a rather dangerous factor for vital organs tissue damage (Chua et al. 2014; Cremieux et al. 2014, Tang et al. 2014), and play a vital role in sepsis, pneumonia, cellulitis and so on. However, RNAIII, as a regulator, may control these virulence genes production and regulates the expression levels of these virulence factors (Frohlich and Vogel 2009; Vanderpool et al. 2011), especially α-hemolysin (Korem et al. 2005; Pang et al. 2010; Tavares et al. 2014). In our studies, RIP was firstly verified to inhibit the RNAIII expression of *S. aureus* isolated from liver tissue of BALB/c mice. Among three RIPs, RIP-V exhibited the strongest inhibitory effect on RNAIII and can decrease markedly the secretion of α-hemolysin in liver. As a quorum-sensing inhibitor, RIP disrupted the signaling process among bacteria and reduced the release of virulence factors (Harraghy et al. 2007). Thus, RIP has little influence on the bacterial survival and reduces the likelihood of resistance developing. Given the increasing restrictions to the clinical application of traditional antibiotics, RIP-V may be a useful anti-infective agent, especially for multidrug-resistant bacteria.

4. Experimental

4.1. Chemicals

Oxacillin and vancomycin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals and reagents used were of analytical grade.

4.2. Organisms

S. aureus ATCC29213 were obtained from the Chinese National Center for Surveillance of Antimicrobial Resistance. MRSA XJ75302 was obtained from the clinical laboratory of Xijing Hospital (Xi'an, China) (Hou et al. 2011).

4.3. Peptide synthesis

RIP-I, RIP-V, RIP-L (Table) were synthesized by the solid-phase method applying Fmoc (9-fluorenylmethyloxycarbonyl) active ester chemistry. All synthesized peptides were purified to over 95% (Table) chromatographic homogeneity by reverse-phase high-performance liquid chromatography. HPLC runs were performed on a C_{18} column with a linear gradient of acetonitrile in water (1% per min), and both solvents contained 0.1% trifluoroacetic acid. Purified RIP powders were identified by mass spectrometer and stored at – 20 °C.

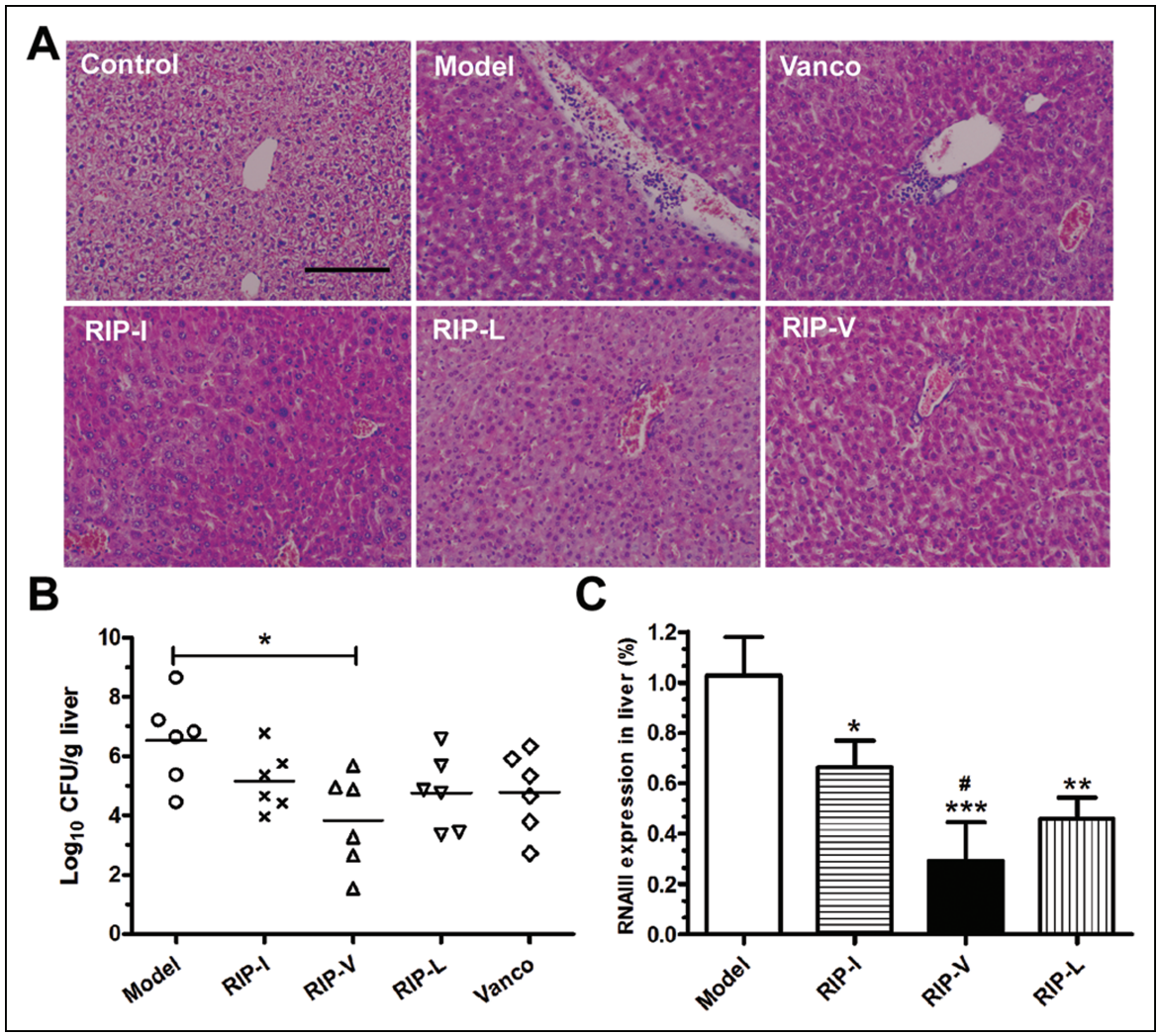

Fig. 5: Protective effect of RIPs on liver in MRSA-infected mice by inhibiting the expression of RNAIII mRNA. BALB/c mice were infected for 1 h and then were administered a single i.p. dose of 10 mg/kg RIP-V, RIP-L, RIP-I or 5 mg/kg vancomycin to evaluate the morphology change in liver. (A) Morphology of the liver was shown with H.E. staining in BALB/c mice infected by MRSA. The original magnification is 200 × , and the bar length represents 100 μm. (B) Colonization of MRSA inoculum in the liver tissue of BALB/c mice after RIP-treated or vancomycin-treated for 8 h (CFUs/g of liver sample). $^{*}P < 0.05$ *vs*. model group. (C) Quantitative RT-PCR analysis of RNAIII mRNA level in MRSA isolated from infected mice liver tissues following treatment with 10 mg/kg different RIPs. RNA expression was quantified after comparison with an internal gene (*16S rRNA*). Data are expressed as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. model group; $^{\#}P < 0.05$ *vs*. RIP-I group.

4.4. Bacterial susceptibility testing and growth assay

S. aureus ATCC29213 and MRSA XJ75302 were grown in nutrient broth with shaking at 37 °C. Bacterial growth was monitored with BIO-RAD680 Microplate Reader ($OD_{600} = 0.1$), which achieved a concentration of approximately 1×10^8 CFU/ml. Antimicrobial susceptibility was determined by a microdilution assay in sterilized 96-well polypropylene microtiter plates. The minimum inhibitory concentration (MIC) was determined according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI).

To determine the growth curve for *S. aureus* ATCC29213 or MRSA XJ75302, synthetic compounds were added to strain cultures to a final concentration of 256 μg/ml. The strains were cultivated in the automated Bioscreen C system (Lab systems Helsinki, Finland), using a Mueller-Hinton (MH) broth culture medium. The working volume in the wells of the Bioscreen plate was 300 μL, comprised of 150 μL of MH broth and 150 μL of drug solution. The temperature was controlled at 35 °C, and the optical density of the cell suspensions was measured automatically at 600 nm in regular intervals of 1 h, for 20 h. Before each measurement, the culture wells were automatically shaken for 60 s. Statistical data for each experiment were obtained from at least two independent assays performed in duplicate.

4.5. MTT assay

The cytotoxicity of RIPs to the human umbilical vein endothelial cells (HUVECs) was determined by a standard MTT assay (Hou et al. 2013). Briefly, cells (5×10^3 cells/well) were maintained in DMEM media with 10% fetal bovine serum (FBS) and grown for 12 h at 37 °C to confluence in a 96-well plate. Then exposed with or without RIPs at various concentrations (0.512–1024 μg/ml) for 48 h. After drug treatment, MTT solution (final concentration, 0.5%) was added and cells were incubated for another 4 h at 37 °C. Dimethyl sulfoxide (DMSO, 150 μl), was added to each well after removal of the supernatant and the absorbance was read at 490 nm.

4.6. Bacterial challenge and RIPs therapy in vivo

Male BALB/c mice, 8–10 weeks old and weighing 18–22 g, were used in our studies. The study was approved by the animal care and use committee of Fourth Military Medical University, Xi'an, China. MRSA was grown overnight in nutrient broth. Mice were infected by the intraperitoneal (i.p.) administration of 5.6×10^8 CFU MRSA inoculum in 0.4 ml of Mueller-Hinton broth. After bacterial challenge for 1 h, mice were randomized to receive i.p. injection with sterile water as a model, 10 mg/kg RIP-V, RIP-L, RIP-I, or 5 mg/kg vancomycin. To assess bacterial clearance, blood samples of six mice in each group were collected from the tail vain and were sacrificed after bacterial challenge for 8 h. Then liver and lung were harvested aseptically from each animal, and then bacterial counts were determined in these tissues. Parts of these tissues were washed with sterile saline, fixed in 10% neutral buffered formalin for 24 h, and then their morphologies were observed using H&E staining. The survival of 11 mice in each group was monitored for 7 days after infection, and the cumulative percentage survival was determined.

4.7. Real time RT-PCR

The *in vivo* RNAIII expression levels were measured by directly extracting the MRSA RNA from the livers of infected BALB/c (Moreira et al. 2010).

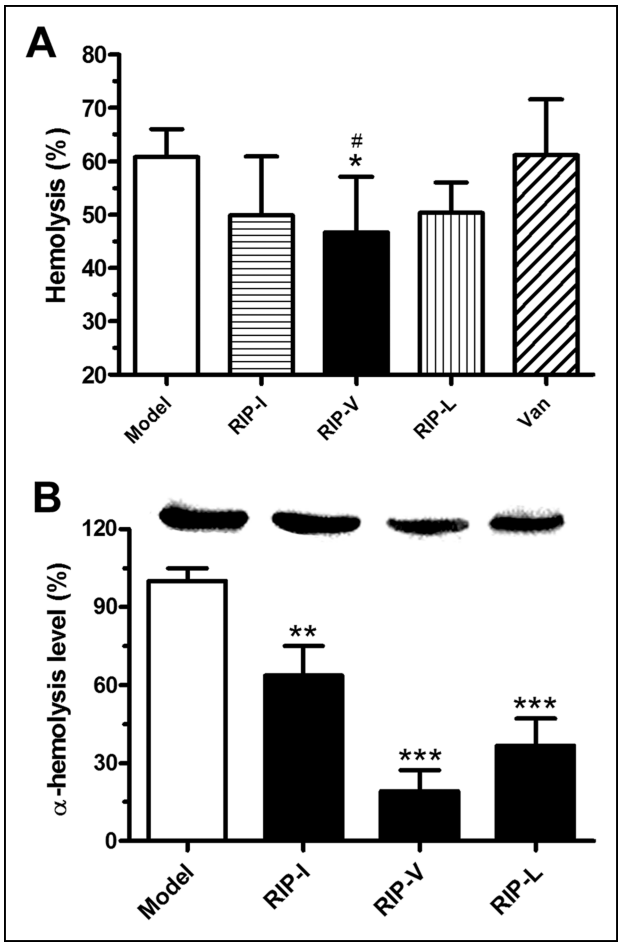

Fig. 6: Inhibition of RIPs on the secretion of α-hemolysin. (A) The level of hemolysins in serum of MRSA-infected mice after RIPs treatment for 8 h. Serum were incubated with 2% mice RBC in PBS for 24 h. $^{*}P < 0.05$ *vs.* model group; $^{\#}P < 0.05$ *vs.* vancomycin treatment group. (B) Analysis of α-hemolysin protein expression in the liver of MRSA-infected mice treated with 10 mg/kg RIPs. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* model group.

The PCR reactions were carried out in a 20 μl volume and contained ABI SYBR® Green PCR Master Mix, as recommended by the manufacturer. The reactions were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). Cycling conditions were as follows: 95 °C for 2 min; 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 40 s. The value of each sample was normalized using the expression of the 16S rRNA housekeeping gene in the same sample. All samples were analyzed in triplicate, the relative expression levels were analyzed by the △△Ct method. The primer sequence of RNAIII primers were 5'-TTCAATGGCACAAGAT-3' and 5'- GTCCAAGGAAACTAAC-3', respectively. And primers for 16S rRNA were 5'-CTTTATGGGATTTGCTTGA-3' and 5'-CTTTATGGGATTTGCTTGA-3', respectively.

4.8. Hemolysis assay

The hemolytic activity of the mouse serum from lethal sepsis was evaluated as follows: Blood of mice was collected from the orbital sinus after infected for 8 h with or without RIPs treatment. Then the blood was separated by centrifuging at approximately 783 × *g* for 20 min. The serum (50 μl) was incubated with 50 μl of 2% suspensions of normal BALB/c mice erythrocytes at 35 °C and 5% CO_2 for 12 h. Release of hemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm. Hemolysis values of 0% and 100% were determined with a spectrophotometer in phosphate-buffered saline solution and 1% Triton X-100, respectively.

4.9. Western blotting

For western blot, liver tissue was homogenated and cells were lysed using RIPA buffer (Pierce, Rockford, IL, USA) with protease inhibitors by sonication and centrifuged with 12,000 × *g*, at 4 °C for 10 min. Equal amounts of proteins were separated on 15% SDS-PAGE and electrotransferred onto PDVF membranes (Invitrogen). Blocked for 1 h with 5% non-fat dried milk in TBS buffer and probed with anti-Staphylococcus alpha hemolysin antibody (Abcam, 1:800) overnight at 4 °C. After washing with TBST buffer (TBS containing 0.05% Tween-20), the membrane was incubated with a horseradish peroxidase-labeled secondary antibody for 2 h at room temperature, and bands were visualized using an enhanced chemiluminescence (ECL) system (Millipore).

4.10. Statistical analysis

Results are expressed as mean values (±SD). A one-way or two-way ANOVA was used to evaluate statistical significance. A probability value of $P < 0.05$ was considered statistically significant.

Acknowledgments: We thank the help from Prof. Xiuli Xu (Xijing Hospital, Shaanxi, China) for clinical strains. This work was supported by grants from the National Natural Science Foundation of China (81273555), the State Project for Essential Drug Research and Development (2013ZX09J13108) and the Innovation Plan of Science and Technology of Shaanxi Province (2013KTCQ03-04).

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