Combination of Antibiotic, Efflux Pump Inhibitor and Autolysis Inhibitor Protects Mice from S. aureus Peritonitis by Inhibiting α-hemolysin-induced MAPKs/NF-κB/NLRP3 Activation

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Abstract
Background: The infection of staphylococcus aureus (S. aureus) is difficult to treat, our aim was to
investigate the antibacterial abilities of a combination of antibiotic cloxacillin, efflux pump inhibitor
thioridazine and autolysis inhibitor tetracycline and its anti-inflammation properties through inhibiting
α-hemolysin induced MAPKs/NF-κB/NLRP3 activation in vitro and in vivo.
Methods: The antibacterial susceptibility test and three-dimensional checkerboard method were
utilized to investigate the synergistic antibacterial activity of the three-drug combination
cloxacillin/thioridazine/tetracycline, α-hemolysin test and scanning electron microscope were used to
assay the inhibition effects of the combination on the secretion of α-hemolysin and membrane-
derived vesicles production from S. aureus, Western blot and pharmacological inhibition assays were
performed to test α-hemolysin -induced MAPKs/NF-κB/NLRP3 activation in macrophage in vitro.
Results: In vitro, the three-drug combination showed synergistic antistaphylococcal activity; the
subinhibitory concentration of the combination significantly inhibited the secretion of α-hemolysin
related to the number of vesicles produced by S. aureus and significantly inhibited the expression of
MAPKs/NF-κB/NLRP3 proteins in the macrophages induced by S. aureus α-hemolysin. In vivo, the drug
combination significantly reduced bacterial colony-forming unit counts of the viscera in mouse
peritonitis model of S. aureus infection, the combination therapy reduced the primary inflammatory
pathology and the release of bacterial-stimulated cytokines such as IL-1β and TNF-α, and inhibited the
expression of MAPKs/NF-κB/NLRP3 proteins in peritoneal macrophage.
Conclusions: Combination of antibiotic, efflux pump inhibitor and autolysis inhibitor owns good
antistaphylococcal activity and significantly inhibit staphylococcal α-hemolysin and its inflammation,
thus, the combination is a novel strategy of antibacterial and anti-inflammation.

1. Introduction
Staphylococcus aureus (S. aureus) infection is characterized by high complications, high recurrence
rate, and strong drug resistance, and is the main cause of invasive infectious diseases(1). α-
hemolysin (Hlα), as the main virulence factor in the infection of S. aureus, can enhance the ability of
S. aureus to destroy the host immune system and cell surface proteins(2). In addition, related studies
have shown that Hlα can be transported through S. aureus secretes membrane-derived vesicles (SMVs)(3), which is also the cause of Hlα diffusion. In summary, to solve the S. aureus-caused diseases, research should be conducted on the proinflammatory pathogenicity of Hlα and the production of vesicles.

Remarkably, S. aureus Hlα have been reported could induce cell death through an NLRP3-dependent cellular necrosis program, resulting in the release of endogenous pro-inflammatory molecules(4). In addition, mitogen-activated protein kinases (MAPKs) are kinases involved in basic biological processes and expressing cellular responses to external stress signals, MAPKs (JNK, ERK1/2 and P38) regulate inflammatory and immune responses in macrophages(5). Moreover, activation of NF-κB pathway-related factors plays an important role in regulating inflammation(6). Thus, it is very important to make clear the possible sequence relation of MAPKs, NF-κB and NLRP3 in S. aureus Hlα-induced inflammation and find its related inhibitors.

Combination therapy is an effective strategy to deal with refractory bacterial infection(7). Cloxacillin (CXN) is a beta-lactam drug that can be used to treat related infections caused by S. aureus (8). Thioridazine (TZ) is an efflux pump inhibitor that inhibits the bacterial efflux pump genes to some extent(9). Tetracycline (TC) is a broad-spectrum bacteriostatic agent and an autolysis inhibitor(10, 11). Therefore, we set out to develop a new type of three-drug prescription consist, who owns excelent antibacterial and anti-inflammation properties in vitro and in vivo, and its anti-inflammation may be mediated through inhibiting Hlα-induced MAPKs/NF-κB/NLRP3 activation.

2. Materials And Method

2.1 Ethics statement

The mice were housed in isolation cages and given food and water in a timely manner. The ambient temperature is 24 ± 1°C and the relative humidity is 40 - 80%. All animal studies were conducted in accordance with experimental practices and standards approved by the Animal Welfare and Research Ethics Committee of Jilin University (No. IZ-2009-008).

2.2 Cell and strain growth condition

S. aureus 8325-4, DU1090 (isogenic Hla deletion mutant) was kept in the laboratory of the Institute of
Veterinary Diseases, College of Veterinary Medicine, Jilin University. Macrophage RAW264.7, purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). CXN, TZ and TC were purchased from Sigma-Aldrich and dissolved in sterile water at a concentration of 40,960 μg/ml under sterile conditions and stored at -20°C until use.

2.3 Antibacterial susceptibility test

To determine the minimum inhibitory concentration (MIC) of 8325-4, DU1090 versus CXN, TZ and TC, a micro-broth dilution method was used according to the CLSI (formerly known as NCCLS) guidelines. Synergistic assays were performed in 96-well microtiter plates containing two antibacterial agents distributed along the transverse and longitudinal wells in a checkerboard pattern on a dioptric dilution basis. Each well contained 0.1 mL of a separate antimicrobial composition or broth control. Other conditions are the same as the MIC measurement conditions to determine the combined drug results. One drug concentration was fixed when evaluating three antibacterial drug combinations, and the other two drugs were operated as above. The in vitro graded inhibitory concentration index (FICI) of each group was calculated(12). The sum of the three drugs synergistically FICI (ΣFIC) was determined as follows: synergy (ΣFIC ≤ 1), additive effect (ΣFIC = 1), irrelevant effect (ΣFIC = 1 - 4), antagonism (ΣFIC > 4)(13).

2.4 Hemolysis test

*S. aureus* 8325-4/DU1090 was incubated in tryptic soy broth (TSB) at 37°C to the exponential growth phase (OD 600 nm = 2.5) with or without drug or drug combination. The culture was centrifuged and the supernatant was collected. 100 μl containing different concentrations of supernatant were pre-incubated in Eppendorf tubes with defibrin rabbit red blood cells (25 μL) and 875 μl hemolysis buffer (20 mM CaCl₂, 0.125 M NaCl) for 30 min at 37°C. Hemolysis buffer was used as a negative control. After centrifugation, the supernatant was removed and the absorbance was measured at 450 nm. *S. aureus* strain 8325-4/DU1090 was incubated in tryptic soy broth (TSB) at 37°C to the exponential growth phase (OD 600 nm = 2.5) with or without drug combination. The culture was centrifuged at 8,500 g for 30 min at 4°C, and the culture supernatant (900 μL) was incubated with 100 μL of LTCA
for 12 h at 4°C. The supernatant protein was collected and centrifuged at 8,500 g for 70 min at 4°C. The extracted SMVs is then repeatedly frozen and thawed until broken. Protein Hlα was tested by Western blot analysis. The primary antibody recognizing Hlα was purchased from Sigma-Aldrich.

2.5 Scanning electron microscope.

Scanning electron microscopy (SEM) was used to analyze the number of spherical vesicles associated with the surface of S. aureus. Fixation of the cells should be fixed with 2.5% glutaraldehyde for 30 minutes at -4°C. Then, fixed with 1% osmium tetroxide at -4°C for 30 min, dehydrated with a graded ethanol series, dried at a critical point, and covered with a gold film by sputtering. The sample was then analyzed by a scanning electron microscope.

2.6 Vesicle extraction separation

S. aureus 8325-4/DU1090 was incubated with drug-pretreated S. aureus 8325-4/DU1090. It was overnight at 37°C at 200 rpm in an orbital shaker. The S. aureus strain was grown in 200 ml of LB medium and grown to an optical density (OD 600) of 2.0. Then, after a series of gradient centrifugation and filtration, the remaining supernatant was passed through a Beckman cryogenic ultracentrifuge (Beckman, USA) at 100,000 g for ultra-centrifugation at 4°C for 1 h to obtain membrane vesicles.

2.7 Western blot analysis

Bubble-treated RAW264.7 cells (1 × 10^6) obtained from S. aureus 8325-4/DU1090 (E:T ratio, 5:1) or pure Hlα (100 μg/ml, Sigma Aldrich) or ultracentrifuge. The drug combination (CXN: 0.015625 μg/ml, TZ: 0.25 μg/ml, TC: 0.03125 μg/ml) was present or not at 37°C for 6 h. Total protein was collected and the total protein concentration was determined by the BCA method. The same amount of protein was subjected to SDS-PAGE gel electrophoresis, and an ECL imaging system was used for image acquisition.

2.8 Mouse S. aureus peritonitis model

We used the previously described and widely used mouse peritonitis model to measure in vivo antibacterial effects(14, 15). In short, 6-week-old BALB/c female mice were inoculated with resuspended S. aureus (per 50 μL 2 × 10^8 colony-forming unit (CFU). Each experimental group
contained 30 mice. To further investigate the therapeutic effect of the drug combination in vivo, 100 μL of drug or PBS was administered subcutaneously at 2 hours after infection with S. aureus 8325-4 / DU1090, and then administered at 12 hour intervals. After 72 h, the infected mice were euthanized. In vivo, the concentration of the drug combination treatment group was: CXN (1.6125 mg/kg/d), TC (3.125 mg/kg/d), TZ (25 mg/kg/d). Peritoneal macrophages were extracted and counted. Ascites colony counts of each group were performed. For histopathological analysis, formalin-fixed liver and spleen lung tissues were stained with hematoxylin and eosin and visualized using an Olympus BX53 fluorescence microscope with a 20 × objective. Western blot analysis of protein extracted from peritoneal macrophages.

2.9 Statistical analysis

Comparisons of mean values from three experiments were statistically evaluated by analysis of variance, followed by One-Way ANOVA analysis. All statistical analyses were performed using SPSS software (version 11.5; SPSS).

3. Results

3.1 Drug MIC and FICI measurement results

To test the respective MICs of drug CXN, TZ and TC against S. aureus strains in vitro, we conducted a drug sensitivity test. The results showed that the MICs of CXN against S. aureus strain 8325-4 and DU1090 (an H1α-deleted strain) were 0.125 μg/mL and 0.125 μg/mL respectively; the MICs of TZ against strain 8325-4 and DU1090 were 16 μg/mL and 32 μg/mL, respectively; the MICs of TC against strain 8325-4 and DU1090 were 0.125 μg/mL and 0.125 μg/mL, respectively (Table 1). To test the synergistic antibacterial activity of combination CXN, TZ and TC in vitro, we conducted a three-dimensional checkerboard method. The FICI results of the drug combination showed the two-drug combination of a certain concentration of CXN and TC, CXN and TZ, TZ and TC showed an additive effect on strain 8325-4, DU1090 (Table 2). Whereas the three-drug combination of a certain concentration of CXN, TZ and TC showed synergistic effect on strain 8325-4, and DU1090 (Table 2), for their sum FICI is less than 1.0 [19, 20], among them, we chose the synergistic three-drug combination of MICCXN: MICTZ: MICTC = 0.03125 μg/mL: 0.0625 μg/mL: 0.5 μg/mL as a candidate for
further screen of three-drug prescription having simultaneous antibacterial and hemolysis-inhibiting activities.

3.2 Subinhibitory concentration of three-drug combination inhibits hemolytic activity of Hlα in vitro

To test the effect of drug combination on S. aureus Hlα production, but not inhibiting the bacterial growth, we selected a subinhibitory concentration of three-drug combination (1/2 MICₚₓₓₙ: 1/2 MICₜₚ: 1/2 MICₜ = 0.015625 μg/mL: 0.03125 μg/mL: 0.25 μg/mL) for further experiments including Western blotting, hemolytic activity, and so on. Western blot analysis showed that CXN, TZ or TC alone at the concentration of subinhibitory three-drug combination inhibited the expression of Hlα of S. aureus strain 8325-4 strain to varying degrees, the subinhibitory concentration of three-drug combination of CXN, TZ and TC significantly inhibited the expression of Hlα (Fig. 1A - B). Further blood plate experiment also verified that subinhibitory concentration of three-drug combination that of CXN, TZ and TC had best hemolysis-inhibiting activities (Fig. 1E). Moreover, By measuring the absorbance of the supernatant at 450 nm with a spectrophotometer (Fig. 1C - D), we found that CXN, TZ or TC alone had a certain of hemolysis-inhibiting activities, we also found subinhibitory concentration of three-drug combination had best hemolysis-inhibiting effect.

3.3 Subinhibitory concentration of three-drug combination inhibits the production of SMVs

Recent research evidence indicates that S. aureus secretes Hlα-containing SMVs to the outside of the cell, and Hlα is closely related to SMVs. In our study, we observed S. aureus strain 8325-4 produced much more SMVs on the surface than that of strain DU1090, whereas the drug CXN, TZ or TC alone inhibited the production of SMVs to varying degrees, especially, the subinhibitory concentration of three-drug combination treatment significantly inhibited the production of vesicles in strain 8325-4 than that of drugs alone treatment (Fig. 2A, 2D). In addition, we compared the expression of Hlα in the SMVs extracted from strain 8325-4 treated by different drug treatments, the results showed that Hlα was expressed in the SMVs isolated from strain 8325-4, and the subinhibitory concentration of three-drug combination significantly inhibited the expression of Hlα, however, the expression of Hlα in
SMVs was not found in strain DU1090 (Fig. 2B - C). These results suggest that Hlα is associated with SMVs, and that the subinhibitory concentration of three-drug combination suppresses the production of SMVs and the expression of Hlα mediated by SMVs.

3.4 Subinhibitory concentration of three-drug combination inhibits MAPKs, NF-κB and NLRP3 pathways in macrophage induced by S. aureus, SMVs or Hlα

In order to detect the effects of the three-drug combinations on the inflammatory pathway in S. aureus, Hlα or vesicle-induced RAW264.7 cells, we performed Western blot analysis on the expression of MAPKs (Fig. 3A), NF-κB (Fig. 3B) and NLRP3 (Fig. 3C) related proteins in the macrophages. The results showed that the expression levels of p-JNK, p-ERK1/2, p-P38, NF-κB, IKK, ASC, IL-18, IL-1β, NLRP3 and caspase-1 were significantly increased in strain 8325-4, 8325-4 MVs, and Hlα inducement group compared with that of the blank control group. However, the expression levels of p-JNK, p-ERK1/2, p-P38, NF-κB, IKK, ASC, IL-18, IL-1β, NLRP3 and caspase-1 were significantly downregulated in the strain DU1090 inducement groups compared with that of strain 8325-4, 8325-4 MVs or Hlα groups. After the subinhibitory concentration of three-drug combination treatment, the expression levels of p-JNK, p-ERK1/2, p-P38, NF-κB, IKK, ASC, IL-18, IL-1β, NLRP3 and caspase-1 were significantly reduced in strain 8325-4, 8325-4 MVs or Hlα groups compared with that of no drug treatment. Noteworthily, the expression of IkB were significantly lower in the strain 8325-4, 8325-4 MVs and Hlα inducement groups than that of the blank control group, the strain DU1090 inducement group, and three-drug combination treatment group. These results indicates that the subinhibitory concentration of three-drug combination of CXN, TZ and TC can inhibit the inflammatory response of MAPKs, NF-κB and NLRP3 pathways induced by S. aureus, vesicle and Hlα in the macrophages in vitro.

3.5 MAPKs (P38 and ERK1/2) mediates Hlα-stimulated activation of NF-κB and NLRP3 inflammasome in macrophage

Further experiments were performed to examine the upstream and downstream relationship between the MAPKs, NF-κB and NLRP3 in the Hlα-activated inflammation pathways, we tested the expression of each pathway proteins in macrophages treated with Hlα under pretreatment using the P38 inhibitor SB 203580, the ERK1/2 inhibitor PD 98059 and the JNKs inhibitor SP 600125, the NF-κB inhibitor BAY
11-7082, and the NLRP3 inhibitor Ac-YVAD-cmk. First, for MAPKs pathway, p38 inhibitor SB 203580, ERK1/2 inhibitor PD98059 and JNK inhibitor SP 600125 inhibited the expression of p-p38, p-ERK and p-JNK, respectively, in addition, the JNK inhibitor SP600125 inhibited the expression of p-p38, p-ERK, and p-JNK, but the p38 inhibitor SP203580 could not inhibit the expression of p-ERK and p-JNK; the ERK1/2 inhibitor PD98059 could not inhibit the expression of p-JNK. These results indicate that JNK is located upstream of ERK1/2 and P38 and that P38 is located downstream of ERK1/2. Second, we found that p38 inhibitor SB203580 and ERK1/2 inhibitor PD98059 instead JNK inhibitor SP600125 significantly inhibited the production of NF-κB, IKK, ASC, IL-18, IL-1β, NLRP3 and caspase-1 (Fig. 4). These results indicate that S. aureus Hlα activated MAPKs (P38, ERK1/2, and JNK), NF-κB and NLRP3 pathways, P38 MAPK and ERK1/2 MAPKs (ERK1/2 located upstream of P38), but not JNK MAPK, mediated NF-κB and NLRP3 pathways.

3.6 Three-drug combination treatment protects mice from S. aureus peritonitis in vivo

As shown above, three-drug combination of CXN, TZ and TC exhibited an excellent antistaphylococcal activity and anti-Hlα-activated inflammation in vitro. Next, we investigated the effect of three-drug combination protecting mice from S. aureus peritonitis in vivo. Gross pathological images, the results showed that the lung tissue of S. aureus strain 8325-4-infected mice showed deep pink with bleeding points, spleen tissue of strain 8325-4 infected mice was swollen, whereas the correspondent inflammation caused by strain DU1090 is weaker than its parental strain 8325-4 (Fig. 5A), and the correspondent inflammation caused by strain DU1090 was less than that of strain 8325-4. Especially, after three-drug combination treatment, the pathological changes of lung tissue and spleen tissue by S. aureus 8325-4-infected are weakened. However, the overall pathological changes of liver tissue were not obvious whatever in mice infected by strain 8325-4, strain DU1090 or treated by three-drug combination. The S. aureus CFU counts in the ascites cell supernatant were taken, and it was found that after three-drug combination treatment, the S. aureus CFU counts were decreased in some extent at 72 h in the peritoneal cavity, however, S. aureus CFU counts was significantly reduced at 144 h (Fig. 5B). Peritoneal macrophage counts showed that the number of macrophages in strain 8325-4 / DU1090-infected mice was significantly higher than that of no infection group, and the
increase was significantly decreased after three-drug combination treatment (Fig. 5C).

Next, we performed Western blot analysis on the extracted mouse peritoneal macrophages (Fig. 6A). The results showed that the three-drug combination significantly reduced the levels of p-JNK, p-ERK1/2, p-P38, NF-κB, IKK, ASC, IL-1β, IL-1β, NLRP3 and caspase-1 (Fig. 6B). Serum ELISA analysis showed that the expression of IL-1β and the expression of TNF-α in the serum of the 8325-4-infected group were higher than those in strain DU1090-infected group and the blank group at 72 h and at 144 h, respectively, and the values of strain DU1090-infected group were higher than those in the blank group, however, the counterpart of the three-drug combination treatment were significantly decreased in strain 8325-4-infected group and in strain DU1090-infected group (Fig. 6C - F). All these results indicate that three-drug combination of CXN, TZ and TC has a good protective effect on mouse pneumonia caused by S. aureus Hlα in vivo.

4. Discussion
A number of studies have shown that combined treatment can not only improve the antibacterial effect, but also reduce the toxicity caused by excessive concentration of a single drug(16). In this study, a three-drug combination consist of CXN, TZ and TC, was used to develop an efficient antistaphylococcal prescription. The results of this study indicate that the combination of CXN, TZ and TC has a synergistic effect against S. aureus (Table 1, 2), thus, this new three-drug combination CXN/TZ/TC can also open a new direction for the treatment of other bacterial diseases.

S. aureus produces potent hemolysins(17) and leukotoxins. S. aureus secretes membrane-derived vesicles (SMVs) that deliver virulence factors to host cells and induce cytopathology(18). Thus, the screen of anti-Hlα drugs is of great importance for the elimination of bacterial toxicity. The results of this study show that the three-drug combination can effectively inhibit the expression of Hlα at a lower concentration than the single drug, indicating that the three-drug combination can effectively reduce the risk of diseases caused by S. aureus Hlα. The drug combination inhibits the hemolytic activity and bacterial protein expression levels of Hlα at concentrations below the MIC (Fig. 1), all these results indicate that the three-drug combination used here inhibits the bacteria and simultaneously inhibits the expression of the bacterial virulence protein Hlα.
Studies have shown that the expression of MAPKs/NF-κB/NLRP3 pathway protein activity is a potential target for the study of anti-inflammatory drugs (19-21). Until now, there were few studies reported the relationship between *S. aureus* Hlα-induced inflammation and simultaneous activation of MAPKs NF-κB and NLRP3. The results of this study demonstrated that the three-drug combination can significantly reduce the phosphorylation of MAPKs protein (JNK ERK1/2 and P38) in *S. aureus*-infected macrophages. The three-drug combination can also significantly reduce NF-κB and NLPR3-related inflammatory proteins in *S. aureus*-infected macrophages. Our results indicate that JNK is located upstream of ERK1/2 and P38, and P38 is located downstream of ERK1/2. In addition, P38 and p-ERK1/2 are located upstream of NF-κB and NLRP3. Taken together, the results indicate that the drug combination inhibits MAPKs-mediated NF-κB and NLRP3 pathways activated by *S. aureus* or SMVs, Hlα infection. This result indicates that the three-drug combination can exert its anti-inflammatory effect through MAPKs, NF-κB and NLPR3 pathways.

In the *in vivo* experiment, the therapeutic effect against mouse peritonitis models obtained by the three-drug combination is remarkable and the results of ELISA analysis of serum also showed a significant decrease of the inflammatory factors IL-1β expression and TNF-α after three-drug combination treatment. All these results indicate that drug has a good protective effect against mouse peritonitis caused by *S. aureus* Hlα *in vivo*.

**Declaration**

**Ethics approval and consent to participate**

The mice in this experiment live in miniature isolation cages and ensure an adequate, healthy diet and water. The laboratory temperature was maintained at 24 ± 1°C and the relative humidity was maintained at 40 ~ 80%. All animal experiment criteria were based on the criteria for laboratory animal welfare and animal approval by the Research Ethics Committee of Jilin University (No. IZ-2009-008). The experimental protocol was reviewed and approved by the committee. In order to minimize animal suffering, all animals were tested under isoflurane anesthesia.

**Consent for publication**

Not applicable.
Availability of data and material

Please contact author for data requests.

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Author's contribution

Lu Yu participated in research design.

Wenjing Luan and Xuefei Wang conducted experiments.

Chao Wang, Lihui Ni and Hong Zhou constructed mice model.

Yanan An and Fangxue Ma performed data analysis.

Yang Wang made Figures.

Lu Yu and Wenjing Luan wrote or contributed to the writing of the manuscript.

Xiaolei Liu, Mingyuan Liu and Lu Yu guided all the aspects of the study.

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Competing Interests

The authors declare no conflicts of interests.

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Tables
Due to technical limitations, the tables are only available as a download in the supplemental files section.

Figures
Figure 1. Subinhibitory concentration of three-drug combination inhibits the hemolytic activity of Hla in vitro.

A

Hla

B

C

D

E
Subinhibitory concentration of three-drug combination inhibits the hemolytic activity of Hla in vitro. (A) Western blot analysis of expression of S. aureus strain 8325-4 in single drug and three drug combinations. (B) The expression of strain 8325-4 treated with different concentrations of drug and three drug combinations. (C) The hemolysis ability of the strain 8325-4 supernatant of each group of drugs was measured by measuring the absorbance at 450 nm. (D) Percentage of hemolytic activity. (E) Hemolysis ability of strain 8325-4 supernatant measured by rabbit co-cultured red blood cells. (a: 8325-4; b: 8325-4 + cloxacillin; c: 8325-4 + tetracycline; d: 8325-4 + thioridazine; e: 8325-4 + cloxacillin + tetracycline; f: 8325-4 + cloxacillin + thioridazine; g: 8325-4 + tetracycline + thioridazine; h: 8325-4 + cloxacillin + tetracycline + thioridazine; i: DU1090). The data is the standard deviation of the means significantly different from strain 8325-4. * P < 0.05, ** p < 0.01, *** p < 0.0001.
Figure 2. Subinhibitory concentration of three-drug inhibits the production of SMVs in *S. aureus*.

**A**

| 8325-4       | 8325-4+CXN | 8325-4+TC |
|--------------|------------|------------|
| 8325-4+TZ    | 8325-4+CXN+TC | 8325-4+CXN+TZ |
| 8325-4+TC+TZ | 8325-4+CXN+TC+TZ | DU1090         |

**B**

| 8325-4       | 8+CXN     | 8+TC      | 8+TZ      | 8+CXN+TC  | 8+CXN+TZ  | 8+TC+TZ   | 8+CXN+TC+TZ | DU1090          |

**C**

![Graph C]

**D**

![Graph D]
Figure 2

Subinhibitory concentration of three-drug inhibits the production of SMVs in S. aureus. (A) Scanning electron microscope for observation of drug treatment or untreated SMVs yield (15K x). (B) The content of Hlα was detected using a Western blot. (C) The content of Hlα of S. aureus strain 8325-4 treated with different concentrations of drug and three drug combinations. (D) SMVs was extracted and quantified. The data is the standard deviation of the means significantly different from strain 8325-4. * P < 0.05, ** p < 0.01, *** p < 0.0001.
Figure 3. Subinhibitory concentration of three-Drug combination inhibits MAPKs, NF-κB, NLRP3 protein activation in S. aureus-, vesicular- or hla-induced macrophages.

| A          | B                      | C                   |
|------------|-------------------------|---------------------|
| 8325-4     | - + + - CLO+TC+TZ      | - + CXN+TC+TZ      |
| DU1090     | - - - + 8325-4MVs      | - + + -            |
| CXN+TC+TZ  | - + + DU1090MVs        | - - + Hla          |
| p-ERK      | p-ERK                  | p-ERK              |
| p-P38      | p-P38                  | p-P38              |
| p-JNK      | p-JNK                  | p-JNK              |
| NF-κB      | NF-κB                  | NF-κB              |
| IKK        | IKK                    | IKK                |
| IKb        | IKb                    | IKb                |
| ASC        | ASC                    | ASC                |
| IL-18      | IL-18                  | IL-18              |
| IL-1β      | IL-1β                  | IL-1β              |
| NLRP3      | NLRP3                  | NLRP3              |
| Caspase-1  | Caspase-1              | Caspase-1          |
| GAPDH      | GAPDH                  | GAPDH              |
Subinhibitory concentration of three-Drug combination inhibits MAPKs, NF-κB, NLRP3 protein activation in S. aureus-, vesicular- or hla-induced macrophages. (A) Western blot was used to test the protein expression of MAPKs, NF-κB and NLRP3 in macrophages induced by S. aureus strain 8325-4 and DU1090 infection. (B) Western blot was used to test the protein expression of MAPKs, NF-κB and NLRP3 in the macrophages treated with the MVs of strain 8325-4 / DU1090 or by drug pretreatment. (C) Western blotting was used to analyze the expression of MAPKs, NF-κB and NLRP3 proteins in S. aureus hla-infected macrophages. The data is the standard deviation of the means significantly different from strain 8325-4. * P < 0.05, ** p < 0.01, *** p < 0.0001.

**Figure 4. MAPKs mediates activation of NF-κB and NLRP3 inflammasome induced by Hlα in MΦ.**
MAPKs mediates activation of NF-κB and NLRP3 inflammasome induced by Hlα in MΦ.

Upstream and downstream relationship between MAPKs, NF-κB and NLRP3 pathways.

Figure 5. The corresponding indicators for *in vivo* drug combination treatment of mouse peritonitis.

A

| K | 8325-4 | 8325-4+CXN+TC+TZ | DU1090 | DU1090+ CXN+TC+TZ |
|---|--------|------------------|--------|--------------------|

B

| K | 8325-4 | 8325-4+CXN+TC+TZ | DU1090 | DU1090+ CXN+TC+TZ |
Figure 5

(A) Gross pathological images of liver, spleen and lung tissues. (B) Results of colony counts in ascites cell supernatants. (C) Results of colony counts in ascites cell supernatants at 72 h. (D) Results of colony counts in ascites cell supernatants at 144 h. (E) Peritoneal macrophage count results.

Figure 6. Western blot and ELISA analysis on the extracted macrophages.

| A          | B          |
|------------|------------|
| 8325-4     | - + + - -  |
| DU1090     | - - + + +  |
| CXN+TC+TZ  | - + - + -  |

| p-ERK      |             |
|------------|-------------|
| p-P38      |             |
| p-JNK      |             |
| IKK        |             |
| IKb        |             |
| NF-κB      |             |
| Caspase-1  |             |
Figure 6

(A) Expression of peritoneal macrophage-associated inflammatory protein in the 72 h batch.

(B) Expression of peritoneal macrophage-associated inflammatory protein in 144 h batch.

(C) Expression of IL-1β in serum of the 72 h batch. (D) Expression of TNF-α in serum of the 72 h batch. (E) Expression of IL-1β in serum of the 144 h batch. (F) Expression of TNF-α in serum of the 144 h batch.

Supplementary Files

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