

Prenylated flavonoids combat metabolically dormant methicillin-resistant *Staphylococcus aureus* by targeting FarR

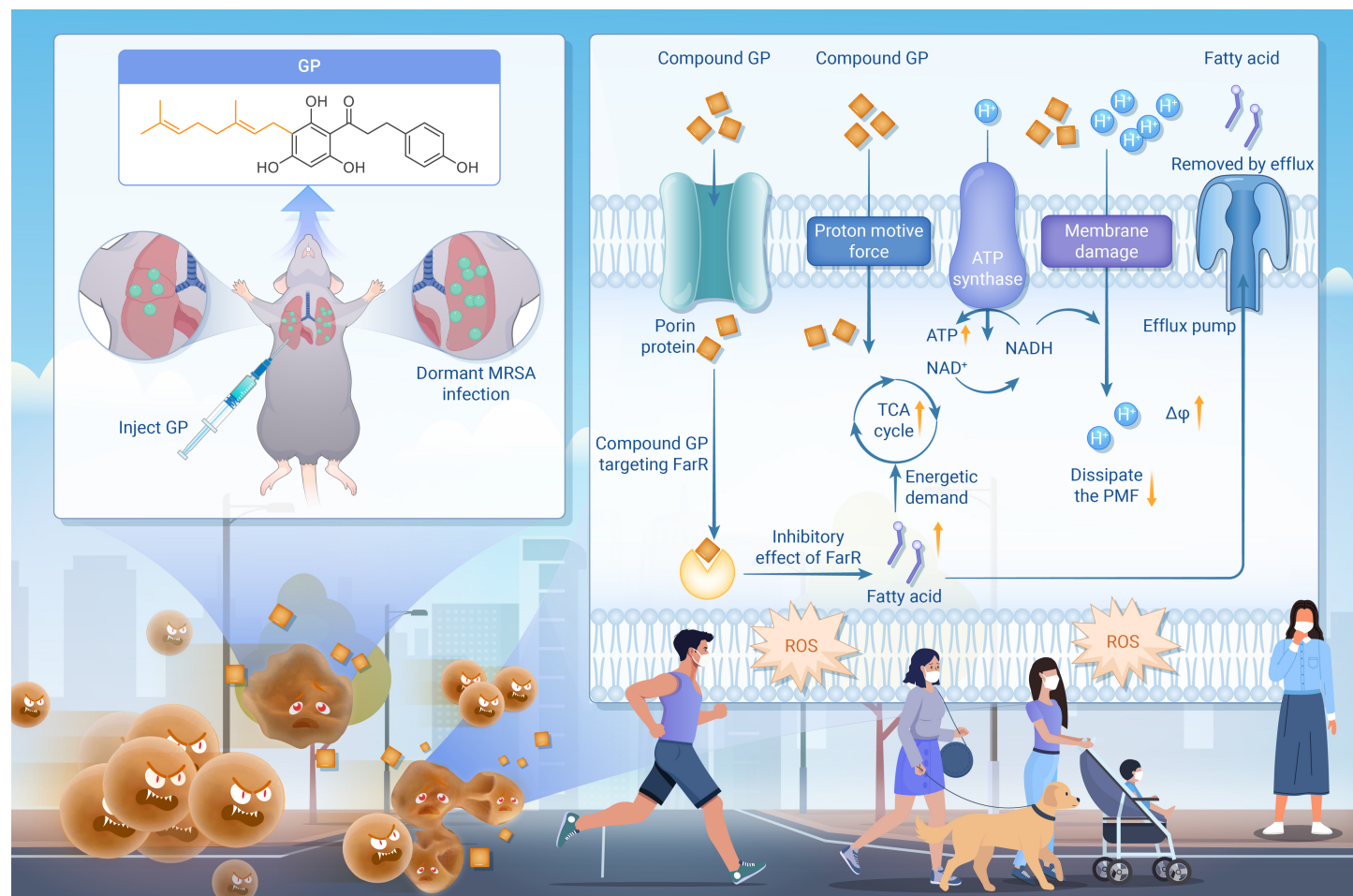
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- A total of 44 prenylated flavonoids were evaluated for antimicrobial activity.
- 3'-C-geranylphloretin (GP) kills multi drug resistant *S. aureus* (MRSA) persister and induces apoptosis-like cell death.
- GP activates the TCA cycle in *S. aureus*, disrupting fatty acid metabolism. FarR participates in GP-mediated bactericidal cell death.
- FarR is first reported as a critical regulator governing *S. aureus* persister cell development.

Prenylated flavonoids combat metabolically dormant methicillin-resistant *Staphylococcus aureus* by targeting FarR

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Antibiotic resistance and bacterial persistence threaten global health, with methicillin-resistant *Staphylococcus aureus* (MRSA) being a particularly challenging pathogen. In this work, 3'-C-geranylphloretin (GP), a prenylated phenolic compound, showed better bactericidal activity against dormant MRSA cells than vancomycin. It significantly decreased bacterial loads, lung index, and improved pathological deterioration mediated by MRSA infection *in vivo*, and showed good safety to normal mice. Bacterial clearance was directly observed by two-photon excitation microscopy. GP disrupted bacterial persistence by targeting the transcriptional regulator FarR, which activated fatty acid and energy metabolism. Besides, GP-induced cell membrane disruption and apoptosis-like cell death in MRSA are caused by chromosomal condensation, reactive oxygen species (ROS) accumulation, and caspase-3/7 activation. Our research holds potential for anti-persister drug development.

INTRODUCTION

Staphylococcus aureus (*S. aureus*), a significant human and animal pathogen, poses a global health threat due to its ability to develop antibiotic resistance.¹ Among its resistant strains, methicillin-resistant *Staphylococcus aureus* (MRSA) is particularly concerned. MRSA infections are challenging to treat, as these bacteria evade conventional antibiotics by entering metabolically dormant "persister" states in response to stressors like antimicrobial agents.^{2,3} This dormancy enables persistent infections, contributing to significant morbidity and mortality worldwide.

The global burden of MRSA is profound, with approximately 80,000 annual cases in the United States alone, resulting in nearly 10,000 deaths.⁴ The elderly population, particularly in healthcare settings, faces a heightened risk, with prevalence exceeding 14.69% in geriatric care facilities.⁵ MRSA infections contribute to over 100,000 deaths worldwide each year.⁶ Among MRSA-related conditions, respiratory infections such as pneumonia and lung abscesses are particularly concerning.⁷ The incidence of MRSA pneumonia has significantly increased over the past two decades, now accounting for nearly 30% of hospital-acquired pneumonia cases.^{8,9}

A key challenge in MRSA treatment is the presence of metabolically dormant persister cells, which evade antibiotics and drive recurrent infections.^{10,11} Conventional antibiotics, including β -lactams and glycopeptides, show limited efficacy against these cells, while alternative treatments such as antitumor antibiotics are hindered by toxicity.¹²⁻¹⁴ This underscores the urgent need for safer, more effective therapies.

Prenylated flavonoids, with the distinct structural characteristics, have demonstrated substantial promise in tackling persistent MRSA infections due to their ability to penetrate bacterial biofilms and demonstrate efficacy

against persistent infections.¹⁵ Additionally, their advantageous safety reduces the likelihood of toxicity and side effects, thereby increasing patient adherence.¹⁶ Lastly, the adaptability of these compounds in pharmacological applications reinforces their suitability as potential candidates in the formula of novel anti-MRSA treatments. This study systematically evaluated the antibacterial activity of 44 flavonoids, elucidating their mechanisms of action, and identifying molecular targets in dormant MRSA. The findings highlight the therapeutic potential of prenylated flavonoids as a novel antimicrobial class, offering a critical solution to address the growing issue of antimicrobial resistance in clinical practice.

MATERIALS AND METHODS

Flavonoids preparation

The synthesis of prenylated flavonoid, 3'-C-geranylphloretin (GP), was carried out according to the reference with some modifications.¹⁷ The detailed procedure is provided in the supplemental information.

Strains, plasmids, primers, chemicals, and commercial kits

The detailed information is provided in Table S1.

Bacterial culture conditions and growth curve measurement

Bacterial cells were grown, killed or treated in Luria-Bertani (LB) medium for *in vitro* tests. Bacterial cells were grown in liquid LB medium for infection works in animal model. LB medium containing 1.5% agar was applied to form individual colonies for CFUs enumeration at 37°C statically. Cells were grown aerobically from single colonies at 37°C, 220 rpm with vigorous shaking at a volume of 4 mL in a 14 mL of Falcon tube (Coring, Cat No. 352059). All plasmid contracts are priorly expanded in DH5a. Details of growth curve measurement are provided in the supplemental information.

High-throughput screening

Screening method followed with recent reports with applying on strains including changes from *E. coli* BW25113 to *S. aureus*.¹⁸

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) was determined anaerobically for all the bactericides in all the strains listed in the key resource table following the same procedure. Protocol of MIC assay was referring to previous research with some modifications.¹⁹ The detailed procedure is provided in the supplemental information.

Bacterial killing & Biofilm assay

Details of *in vitro* bacterial killing against exponential-phase, stationary-

phase or bactericide-mediated persister cells, biofilm coated inside PE50 catheter or formed on glass-bottom 6-well plate are provided in supplementary experimental procedures with some modifications from previous study.^{20,21}

Scanning Electron Microscopy (SEM) assay

Samples were observed using a cryo-scanning electron microscope (SEM) (Hitachi SU8100). The detailed procedure is provided in the supplemental information.

Bacterial apoptosis-like cell death (ALD) measurement

Intracellular ATP measurement (Luciferase luminescence), caspase-substrate affinity (FITC-DEVDG-peptide), membrane potential change ($\Delta\Psi$) and ROS accumulation (Carboxy- H_2 DCFDA) are provided in the supplemental information.

Aggresome observation & statistics

The observation, figure capture and reconstruction of bacterial cells with aggresome at single cell level was implemented via a commercial super-resolution microscope imaging system (HIS-SIM; Guangzhou CSR Biotech Co. Ltd). Imaging procedure was performed by following the previous report.²²⁻²⁴ The detailed procedure is provided in the supplemental information.

Targeted metabolomics

Details of sample preparation, targeted energy or fatty acid metabolomics, data acquisition and analysis are provided in the supplementary experimental procedures. Chromatographic separation was performed using an Agilent 1290II ultra-high-pressure liquid chromatography (UHPLC) system equipped with 6546 quadrupole time-of-flight (QTOF) mass spectrometry. The Agilent 5977B GC/MSD equipped with an Agilent 8890 GC and an Agilent 7693A autosampler was used to quantify 37 fatty acid methyl esters (FAMES). The detailed procedure is provided in the supplemental information.

S. aureus GP-resistant strain development assay

Resistance was assessed by sequential passaging, as described previously.^{25,26} The detailed procedure is provided in the supplemental information.

Whole-genome sequencing

Whole-genome sequencing was performed on the Illumina HiSeq2000 platform at Novogene (Novogene, Beijing, China) using MRSA 43300 as the reference strain.

Homology modeling of FarR protein and docking

Details of Molecular Docking is provided in the supplemental information.

Interactions of GP with FarR by bio-layer interferometry (BLI)

BLI experiments were performed as detailed previously.²⁷ The detailed procedure is provided in the supplemental information.

Animals test and ethics

All animal experiments were conducted according to the protocol approved by the Animal Welfare and Research Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (20240039). Details of bacterial infection, treatment and intra-vital imaging of mouse lung are provided in the supplemental information.^{28,29}

Super resolution fluorescent microscope and images processing

ZEISS Elyra 7 with Lattice SIM² super resolution fluorescent microscope (ZEISS, Germany) with 63 $\times$ oil immersion objective and a commercial super-resolution microscope imaging system (HIS-SIM; Guangzhou CSR Biotech Co. Ltd) were applied for imaging capture unless specially indicated. Details of figure capture, reconstruction and fluorescence-based semi-quantification are provided in the supplemental information.

Statistical analyses

All the tests for this study were conducted at least three times. One-way

analysis of variance (ANOVA) is used for statistical analysis, and the data is represented as mean $\pm$ SD. Tukey's test is used for analysis with statistical significance set at $P < 0.05$. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

RESULTS

Prenylated flavonoids eradicates *S. aureus* persister and biofilms *in vitro*

Prenylated flavonoids exhibit significant potential in treating chronic MRSA infections, due to their unique structural features.³⁰ However, systematic investigations spanning phenotypic confirmation to mechanistic discovery remain lacking. Consequently, this study investigated the capacity of prenylated flavonoids to eradicate bacterial persister cells. Primarily, we established a whole cell-based high-throughput screening platform against various bacterial persister, applying forty-four prenylated flavonoids, as illustrated in the Figure 1A.¹⁸ The anti-persister activity of flavonoids 1-44 were tested against several bacteria including *Pseudomonas aeruginosa* PAO1, *Escherichia coli* BW25113, *Acinetobacter baumannii* Ab19606, carbapenem-sensitive *Klebsiella pneumoniae* KP 13883, *Escherichia coli* 25922, *S. aureus* RN4200, *S. aureus* 29213 and MRSA. As illustrated in Figures 1B-G and supplemental information Figure S4, several prenylated flavonoids demonstrated good antibacterial efficacy against MRSA, *S. aureus* 29213, and RN4220. However, these compounds exhibit no effectiveness against the aforementioned Gram-negative bacteria, thereby indicating a selective activity for Gram-positive bacteria. This specificity can be attributed to the structural features of prenylated flavonoids, including their lipophilic isoprenyl side chains. The particular structures enable these compounds to interact with the thick peptidoglycan layer and membrane of Gram-positive bacteria. However, they are unable to penetrate the outer membrane of Gram-negative bacteria, owing to the presence of lipopolysaccharides. Furthermore, we found that the flavonoids with prenyl groups at the C-6 or C-3' position exhibited significant antibacterial effects, particularly for dihydrochalcone (Figures 1H & S5), a noteworthy structural feature.

Among all the tested compounds, the prenylated flavonoid 3'-C-geranylphloretin (GP) exhibited the most significant effect. The structural information of GP and other prenylated flavonoids were listed in Table S2, Figures S1-S3. At 10 μ M, GP significantly inhibited the persister cells of *S. aureus* 29213 and MRSA (Figures 2A-B). As shown in Figures 2C-F, after 24-h treatment with 2 μ M of GP, besides showing strong bactericidal activity against RN4220 in mid-log phase, it also killed the stationary phase of RN4220 and MRSA (MIC < 1 μ M). In comparison to the commonly used antibiotics such as glycopeptide vancomycin (Van), β -lactam antibiotic meropenem (Mpm), aminoglycoside antibiotics gentamicin (Gen) and kanamycin (Kan), third generation quinolone antimicrobials levofloxacin (Lev), ofloxacin (Ofi) and norfloxacin (Nor), semi-synthetic macrolide antibiotic azithromycin (Azm), and antitumor antibiotic mitomycin C (MMC), this unique capability of GP is not possessed by these antibiotics.

Biofilm formation makes the sterilization more difficult and leads to the chronicity and severity of many clinical diseases. Consequently, following confirmation of its specific eradication efficacy against *S. aureus* via high-throughput phenotype screening, this study further evaluated the compound's activity against persister cells and biofilms. As illustrated in Figures 2G-H & S6, MRSA, *S. aureus* 29213, and *S. aureus* RN4220 were pre-formed into biofilms. The results showed that Gen and Nor had no effects on the pre-formed biofilms of MRSA and *S. aureus* 29213 strains. In addition, at certain concentrations, Mpm (25 μ M) not only had no effect, but also promoted the formation of bacterial biofilms. GP significantly eradicated the pre-formed biofilms of MRSA, *S. aureus* 29213 and *S. aureus* RN4220 at various concentrations (Figures 2I-J).

Further analysis showed (Figures 2K-R) that after the treatment of Van, Mpm, Gen and Lev, both *S. aureus* 29213 and MRSA reached to a stasis phase (persister cell state) in the bi-phasic curve. Then, incubation with GP eradicated persister cells formed in response to these antibiotics after 20 h, indicating the activity of GP to eradicate antibiotic-induced persisters and suggesting the possible synergistic use of GP with antibiotics such as Van, Mpm, Gen, and Lev.

Taking together, these result supported that GP was an effective anti-persister chemical against *S. aureus*, even biofilm eradication *in vitro*.

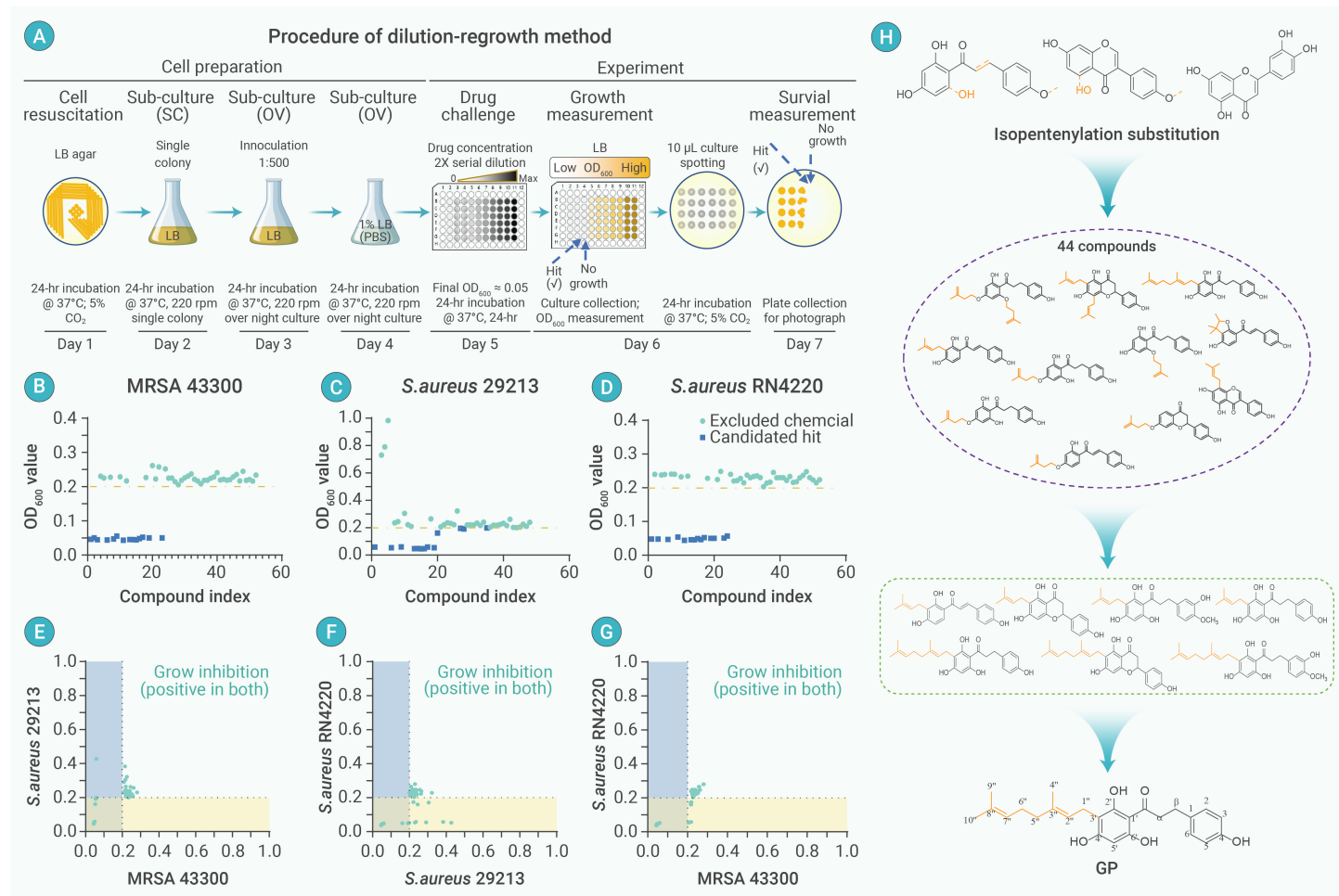


Figure 1. Assessment of the antimicrobial properties of prenylated flavonoids *in vitro* (A) The dilution and regrowth-based screening assay to evaluate the antimicrobial efficacy of isoprenylphenols. (B-D) A normalized OD₆₀₀ value of less than 0.2 was employed as the criterion to differentiate between successful and unsuccessful hits. The section that met this criterion was colored red, while the portion that did not meet the cut-off was colored blue. (E-G) Classifying compounds with antibacterial activity, identifying that simultaneously kill *S. aureus* 29213, *S. aureus* RN4220, and MRSA. All shown are the means of three biological replicates. (H) The chemical structures of flavonoids were screened for activity. This was achieved through the evaluation of 44 obtained compounds. The isoprenyl flavonoids demonstrated prominent activities highlighted in green boxes. These flavonoids share a common prenyl substitutions position on Ring A.

Prenylated flavonoids eradicates MRSA persister *in vivo*

Accordingly, before further study on its mechanism, we aimed to evaluate the *in vivo* efficacy of GP against acute pneumonia induced by MRSA stationary-phase bacteria cell, which is abundant in persister cell and metabolically dormant.³¹ In this study, 6-7 weeks female C57 BL/6J mice were selected and infected with MRSA persister bacteria. Details of the infection model and drug administration procedures are presented in Figure 3A. Besides, to ensure the safety of the chemicals, a toxicity test on the administration concentration of 100 mg/kg/day of GP were conducted for one week, focusing on the liver and kidney. As illustrated in Figures 3I-L, the GP administration concentration of 100 mg/kg/day did not induce any pathological abnormalities in the lung, liver, or kidney, thereby demonstrating its non-toxicity towards major organs.

Furthermore, the lung index and bacterial colony-forming units (CFU) in lung tissues were quantified to assess infection severity and therapeutic efficacy. As shown in Figures 3B-C, MRSA infection significantly increased bacterial load in the lungs. Treatment with the positive control vancomycin (50 mg/kg/day) effectively reduced both the lung index and CFU. Similarly, GP administered at medium (50 mg/kg/day) and high (100 mg/kg/day) doses significantly alleviated lung infection. Importantly, the high-dose GP group demonstrated a more pronounced reduction in lung index compared to the vancomycin-treated group. The results of the histological examinations revealed a distinct trend (Figures 3D-F). Following MRSA persister infection, the alveolar epithelium demonstrated hyperplasia. There was significant infiltration of inflammatory cells and hemorrhage in the lungs, with neutrophils being noticeable in the adjacent lung tissue. However, after treatment with GP

(100 mg/kg), the pathological changes were significantly improved, with only a few inflammatory cells observed.

Beside determination at bulk culture level via CFU enumeration and pathological change, we also analysed the bacterial distribution at single cell level applying *in vivo* two-photon intra-vital imaging method. We made a naive model by in-situ injecting 100 μ L of *S. aureus* RN4220-GFP bacteria suspension (stationary phase, 10⁷ CFU/mL) directly into the mouse left lung. The collagen presents in the lung had a clear second harmonic generation signal (Figures 3G-H). Regarding the infection progression, the bacteria-infected mouse succumbed within approximately one hour. We observed severe lung infection since numerous distinct fluorescent patches from *S. aureus* RN4220-GFP micro-colony in the field of view (Figure 3G). Conversely, the number of fluorescent patches in the lungs of mice receiving only bacteria and drug injections was comparatively lower (Figure 3H), suggesting a less severe infection.

These findings supported GP could be an effective treatment therapy for MRSA lung infections in mice model either at macro- or micro-perspective, implying its developmental potential for next-step study on how it killed bacteria.

S. aureus morphological change and surface deterioration at single cell level

To study how GP acted on *S. aureus*, we started from morphological changes of bacterial cell wall surface at single cell level (micro-perspective). Scanning electron microscopy (SEM) was used to observe the cell surface changes induced by GP or antibiotics. The morphology of untreated *S. aureus*

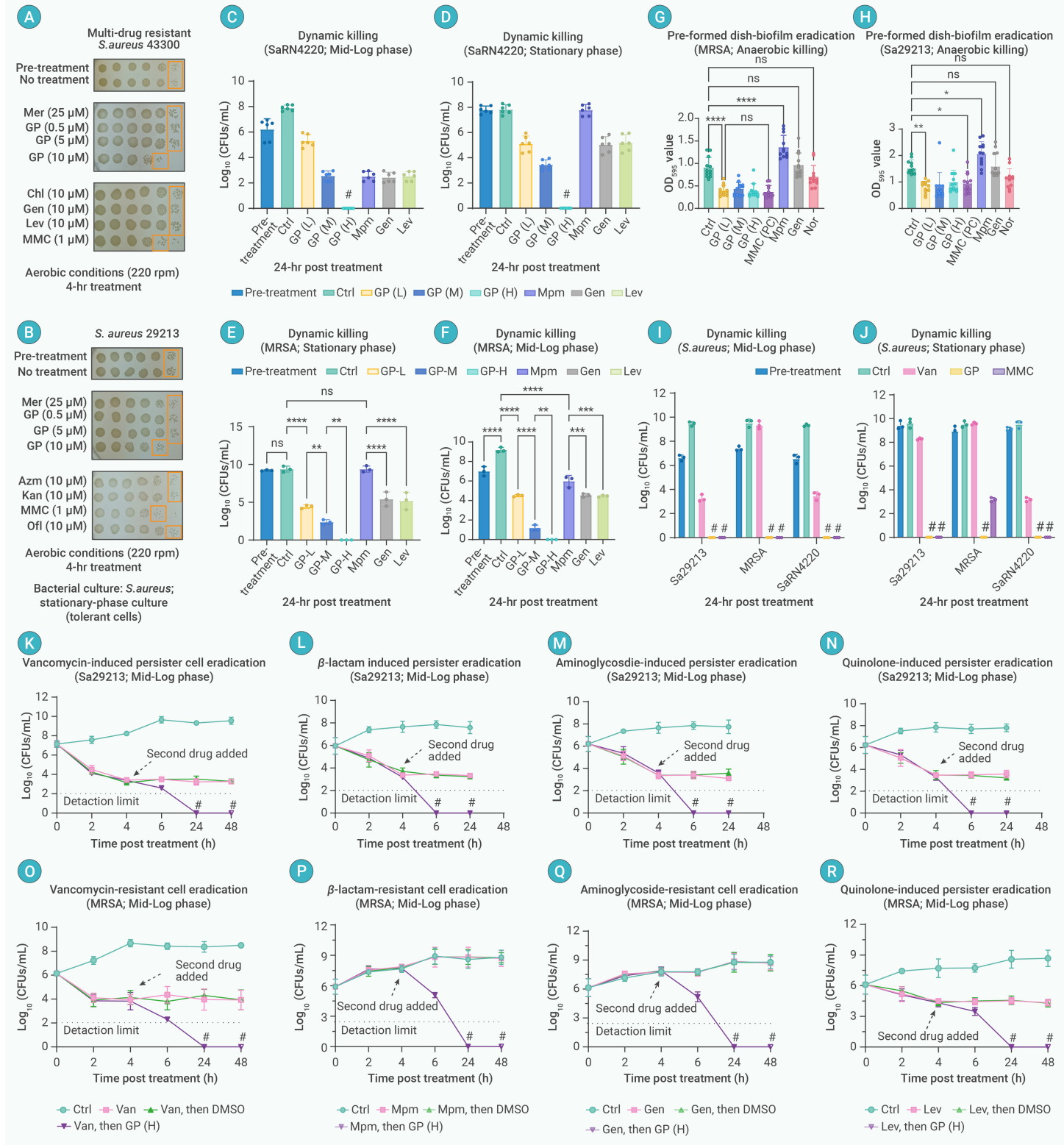


Figure 2. GP kills stationary phase MRSA and *S. aureus* cells directly (A-B) Compound GP kills common antibiotics tolerant *S. aureus* persister in a concentration-dependent manner. (C-F) Bacterial survival enumeration (CFUs) post treated by various bactericides or GP. (G-J) Crystal violet staining of *S. aureus* 29213 (*Sa29213*) pre-formed biofilm survival level. (K-R) Time-dependent killing curve of GP against various bactericide-mediated *S. aureus* persister cells. The antibiotics used employed for testing included vancomycin (Van), meropenem (Mpm), gentamicin (Gen), kanamycin (Kan), levofloxacin (Lev), ofloxacin (Ofi), norfloxacin (Nor), azithromycin (Azm), and mitomycin C (MMC). Data are shown as mean $\pm$ SD ($n \geq 3$). Statistical analysis of CFUs was conducted by application of ordinary One-way ANOVA multiple comparison. ns, no significant difference, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

was characterized by a smooth and uniform spherical shape with intact cell wall and membrane, appearing as grape-like clusters, which was the typical characteristics of normal *S. aureus* cells (Figure 4A). Exposed to MMC and Mpm for one hour, the bacterial surface began to deform, with folds, depres-

sions and ruptures becoming apparent. The cell membrane was apparently loss of integrity causing cellular constituents' leakage. When treated with GP, it was obviously demonstrated that the shape of *S. aureus* cells was altered and distorted with wrinkles and gullies distributed on cell surface. Meanwhile,

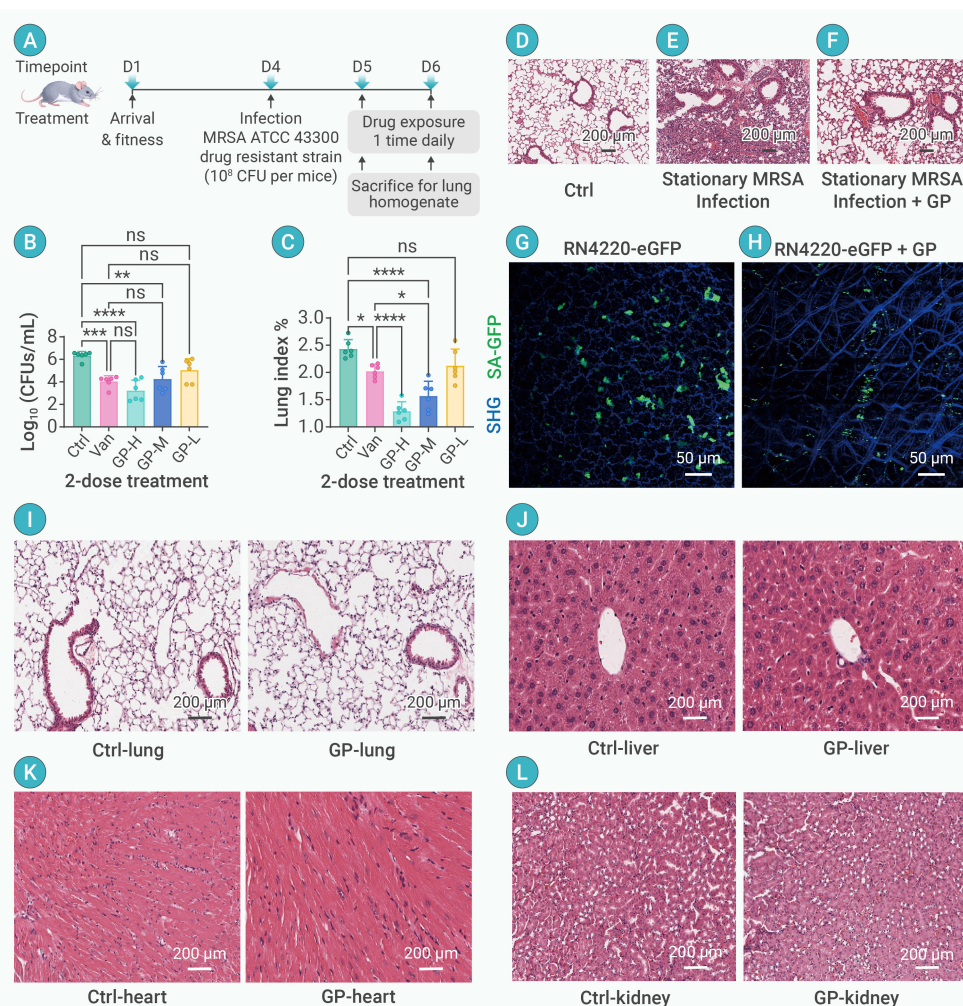


Figure 3. Stationary phase MRSA eradication efficacy of GP *in vivo* (A) Scheme of experimental protocol for testing anti-MRSA activity of GP in C57BL/6J mice. (B) Bacterial burdens in the lungs of various groups following stationary phase MRSA infection. (C) Lung index of various groups following the stationary phase of MRSA infection. (D-F) Histological evaluation of infected lung in different groups. PBS treatment group (D); stationary phase MRSA infection group (E); high-dose GP (100 mg/kg/day) treatment group (F). (G-H) The maximum-intensity-projection Figure via intra-vital lung imaging to visualize the lung infection situation about 1 h after in-situ *S. aureus* RN4220-GFP bacteria injection. the bacteria injection mouse died soon and the lung had been heavily infected (G). The lung of both bacteria and GP injection mouse (H) had milder infection. SHG: second harmonic generation, indicates the collagen in the lung. SA-GFP: the *S. aureus* RN4220-GFP bacteria. (I-L) *In vivo* drug toxicity evaluation of compound GP. Histological evaluation of lung (I), liver (J), heart (K), and kidney (L) after ctrl (PBS treatment) and GP (200 mg/kg/day) treatment. Data are shown as mean $\pm$ SD (n $\geq$ 3). Statistical analysis of Lung Index & CFUs was conducted by application of ordinary One-way ANOVA multiple comparison. ns, no significant difference, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Prenylated flavonoids triggers *S. aureus* apoptosis-like cell death

According to the GP-induced morphological change and membrane permeability, we speculated that GP facilitated apoptotic like cell death on *S. aureus*. Since similar phenotype has been reported in mitomycin C, which kills persister, biofilm and activates ALD.³²⁻³⁴ To confirm this hypothesis, we started from chromosome status observation through super-resolution microscope.

As assumed, after GP treatment, distinct

it was noteworthy to mention that certain cells were gathered into sizable planktonic aggregates, wherein visible cell autolysis was detected. This phenomenon revealed the presence of cell clusters characterized by pores and web-like formations, implying the abandonment on replication and cell cycle arrest (Figure S7). Abolishment in cell division and filament formation are hall marks of apoptotic *E. coli* cells.³² It implied that GP might induce apoptosis-like cell death (ALD) of *S. aureus*.

To further elucidate the disruption of cell membrane and biofilm formation of *S. aureus* by GP, various dyes were utilized for optical imaging. DiBAC4, a membrane potential-sensitive probe, infiltrates depolarized and apoptotic bacterial cells and binds to intracellular components such as proteins or membranes. Fluorescent ascend represents cell membrane depolarization extent and positive cell quantity. A notable increase in cellular fluorescence was observed following GP treatment, compared to the Ctrl group, indicating that GP intensified membrane depolarization in *S. aureus*, thereby enhancing membrane permeability (Figure 4B). It was also confirmed by semi-quantification of fluorescent signal (Figure 4C). To further evaluate the bactericidal effect of GP on the pre-formed biofilm of *S. aureus*, the bacterial membrane integrity post-treatment was analyzed using SYTO/PI dual-staining and Hoechst33342. Once stained, dead bacteria exhibited red fluorescence, while live bacteria displayed green fluorescence. The reconstructed images showing 2D and 3D structure of biofilm (Figure 4D) demonstrated that, following a 3-h incubation with GP-L (12.5 μ M) and GP-H (50 μ M), there was a marked reduction in green fluorescence and a concomitant rise in red fluorescence. This effect was more pronounced than those observed with MMC and Mpm, further indicating that GP could effectively disrupt the bacterial membrane.

In summary, GP exerted bactericidal action by inducing membrane wrinking, fusion, and alterations in permeability, further leading to membrane rupture, perhaps alongside apoptotic cell death phenotypes. These results provided a clear direction on study about how GP killed *S. aureus* at next stage.

chromosomal condensation regions were observed, marked by intense fluorescence foci (Figure 5A). Reactive oxygen species (ROS) production was found to coincide with chromosomal condensation, which indicated ROS accumulation insult facilitated stress against DNA. Similar patterns emerged with MPM and MMC treatments, suggesting that antibiotic-induced stress significantly altered bacterial stress response pathways and metabolic processes. Besides observation at single cell level, fluorescent change at bulk culture of *S. aureus* 29213 and MRSA also supported our finding (Figures 5D-E).

Next, we applied a FITC-conjugated multiple peptides containing DEVDS sequence, which was specifically recognized and digested by active caspase-3/7. Fluorescent signal enhancement represents apoptotic eukaryotic cell number ascend, which is also found applicable in apoptotic *E. coli* cells.³² In this study, treatment of GP facilitated the intracellular fluorescence formation and accumulation, indicating apoptosis among GP-treated *S. aureus* (Figure 5B). Annexin-V staining further revealed the loss of membrane asymmetry, evidenced by phosphatidylserine (PS) externalization on the bacterial membrane (Figure 5C), a hallmark of early apoptosis. Also, semi-quantitative analysis of annexin-v level (Figure 5F) and caspase substrate affinity (Figure 5G) further supported this result at another perspective.

Taking together, these results indicated the involvement of GP in initiating apoptosis-like cell death phenotypes in *S. aureus*. Previous study has discovered that intracellular ATP elevation is an hallmark of early apoptotic phenotype.³⁵ Meanwhile, ATP decrease has been widely accepted as general characteristic alongside *S. aureus* bacterial persister formation and the latest discovered and named bacterial liquid-liquid phase separation (LLPS)-driven organelle, the aggresome, which is critical for bacterial persister survival.^{31,36,37} Therefore, we aimed to investigate if GP facilitates bacterial intracellular ATP level ascend in the future.

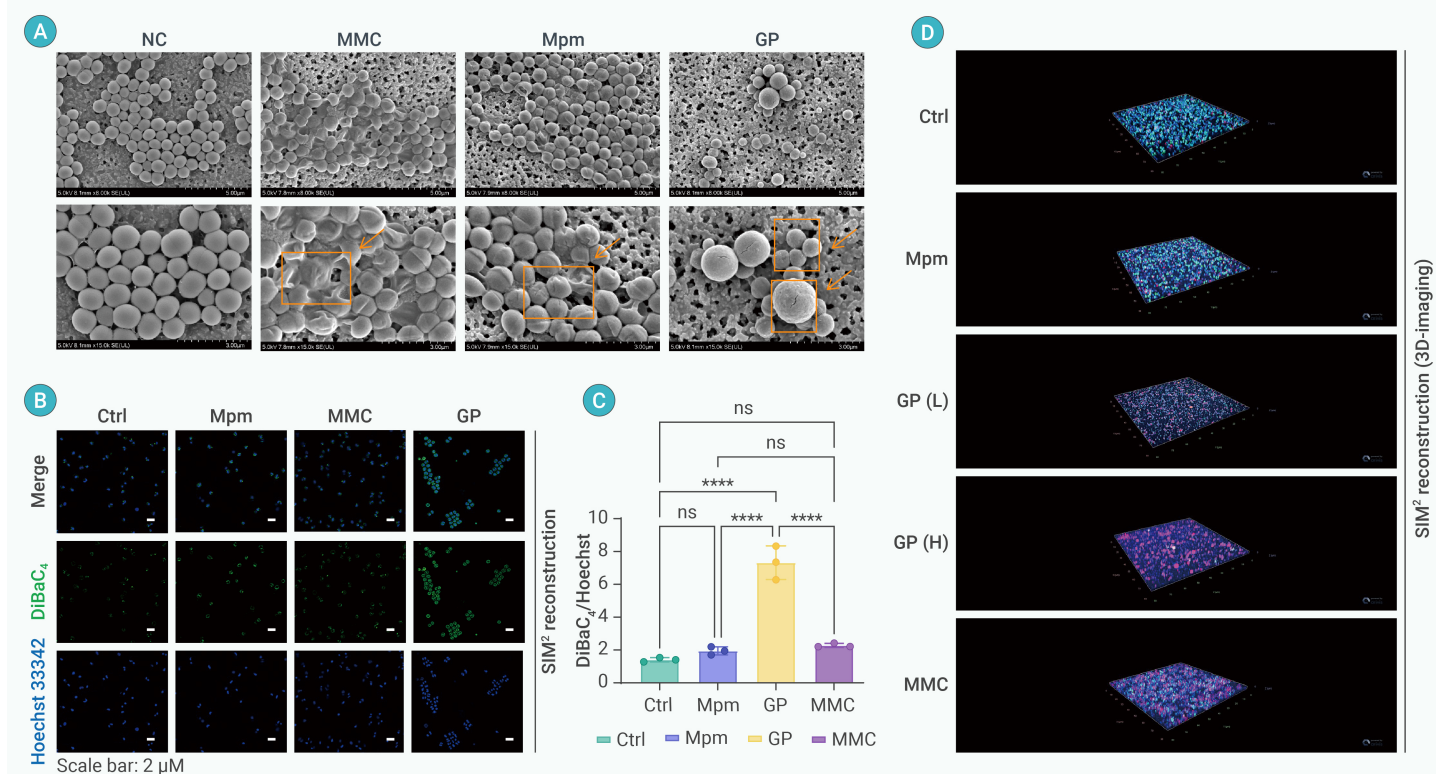


Figure 4. Compound GP directly disrupts the cell membrane of *S. aureus* (A) The scanning electron microscopy images of *S. aureus* under different treatments. The arrow-marked part illustrates cellular distortion and surface damage. (B) The membrane potential of *S. aureus*. (C) The semi-quantitative fluorescence results of the DiBaC₄ to Hoechst33342 ratio. (D) 3D structure images of bacterial biofilm integrity of *S. aureus*. Biofilms are stained with SYTO9 (green, live), PI (red, dead) and Hoechst33342 (blue, chromosome). Data are shown as mean $\pm$ SD ($n \geq 3$). Statistical analysis of Lung Index & CFUs was conducted by application of ordinary One-way ANOVA multiple comparison. ns, no significant difference, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Prenylated flavonoids facilitated intracellular ATP accumulation, energetic metabolism and aggresome decrease

Bacterial protein aggresome, driven by LLPS and ATP depletion, forms dynamic and reversible assemblies under stress conditions. These aggregates contribute to bacterial resilience against various environmental stresses, including antibiotic exposure.³⁷ Since early apoptosis facilitates ATP synthesis, and GP induces ALD and kills persister, we hypothesized GP treatment facilitated intracellular ATP elevation alongside aggresome decrease.³⁵ To clearly observe the bacterial aggresome, we used the latest commercial Structure Illumination Microscopy (SIM) with live cell imaging, provided by HIS-SIM (resolution < 70nm), and figures were processed and reconstructed by the latest sparse deconvolution, which has been peer-reviewed and published (Figure 6A).³⁸

According to the observation, under normal conditions, the sfGFP protein is evenly distributed within bacterial cells, which has been reported.³⁷ In this study, 44 % of *S. aureus* bacterial cells ($N=1099$) at stationary phase were found with distinct fluorescent foci, indicating the formation of protein aggregates. Upon meropenem treatment, aggregate formation was further promoted to enhance bacterial survival under extreme stress, increasing the proportion of fluorescent foci to 59 %. In contrast, treatment with GP and MMC significantly reduced the formation of these aggregates, particularly GP, which lowered the proportion of foci to 33 % (Figure 6B). This phenomenon suggested that GP could impair the formation of protective protein aggregates, which is an important survival strategy option for persister cells to survive antibiotic treatment, ultimately leading to a reduction in bacterial survival under stress.

Previous study reported that protein aggresome formation is driven by intracellular ATP depletion.³⁹ Accordingly, to investigate if GP affected bacterial energetic metabolism, targeted metabolomics were conducted, focusing on central carbon metabolism, amino acid, and nucleotide profiling of GP treated *S. aureus* bacterial cells MRSA and ATCC29213. Each analysis included 8 biological repeats. The PCA analysis revealed significant differences (2-fold changes, $P < 0.05$) in both negative and positive modes for all

the identified metabolites. Our findings highlighted the influence of GP on the metabolome of MRSA and *S. aureus* 29213 (Sa29213), particularly in energy metabolism, as evidenced by relative quantitative targeted metabolome analysis. We analyzed data from both positive and negative modes. Differential and cluster analyses indicated consistent patterns in both modes: metabolites were notably upregulated following treatment with the flavonoid. In the negative mode, specific metabolites associated with the TCA cycle and nucleotides showed the most pronounced increase, as depicted in Figures 6 & S8. Furthermore, NADH, malonyl-CoA, biotin, NADPH, succinyl-CoA, NADP⁺, and NAD⁺ experienced significant elevations. Uracil, glycine, and L-allo-threonine were marginally downregulated in the treated group, whereas other amino acids displayed an upward trend, mirroring the majority of differential metabolites. In contrast, most of differential metabolites in the positive mode were upregulated. Notably, succinate in the TCA cycle, flavine mononucleotide in the pentose phosphate pathway, cAMP in nucleotides, thymidine and uridine in nucleosides exhibited marked reductions in the treatment group. Additionally, L-allo-threonine, aminobutyric acid, dimethylglycine, and creatine in amino acid metabolism were slightly decreased, while betaine in the choline pathway showed a substantial increase. Meanwhile, ATP amount measurement at bulk culture level of Sa29213 and MRSA also supported that GP treatment drives ATP accumulation by a concentration- and time-dependent manner (Figures 6B-C).

Since intracellular ATP depletion and following-by protein aggregation formation or metabolomic dormancy are hall-marks of bacterial persister formation, our results supported that GP lowered number of cells with protein aggregates and up-regulation in central carbon metabolism, implied its significance in mechanism study as an effective anti-persister chemical.

However, the mechanism by which GP treatment simultaneously enhances energy supply and induces ATP depletion remains unclear. Based on observed membrane deterioration, depolarization, and apoptosis initiation, we hypothesized that GP-target interactions disrupted fatty acid metabolism homeostasis. This metabolic dysregulation compromises bacterial membrane integrity and function—critical adaptations for survival in complex environments.⁴⁰

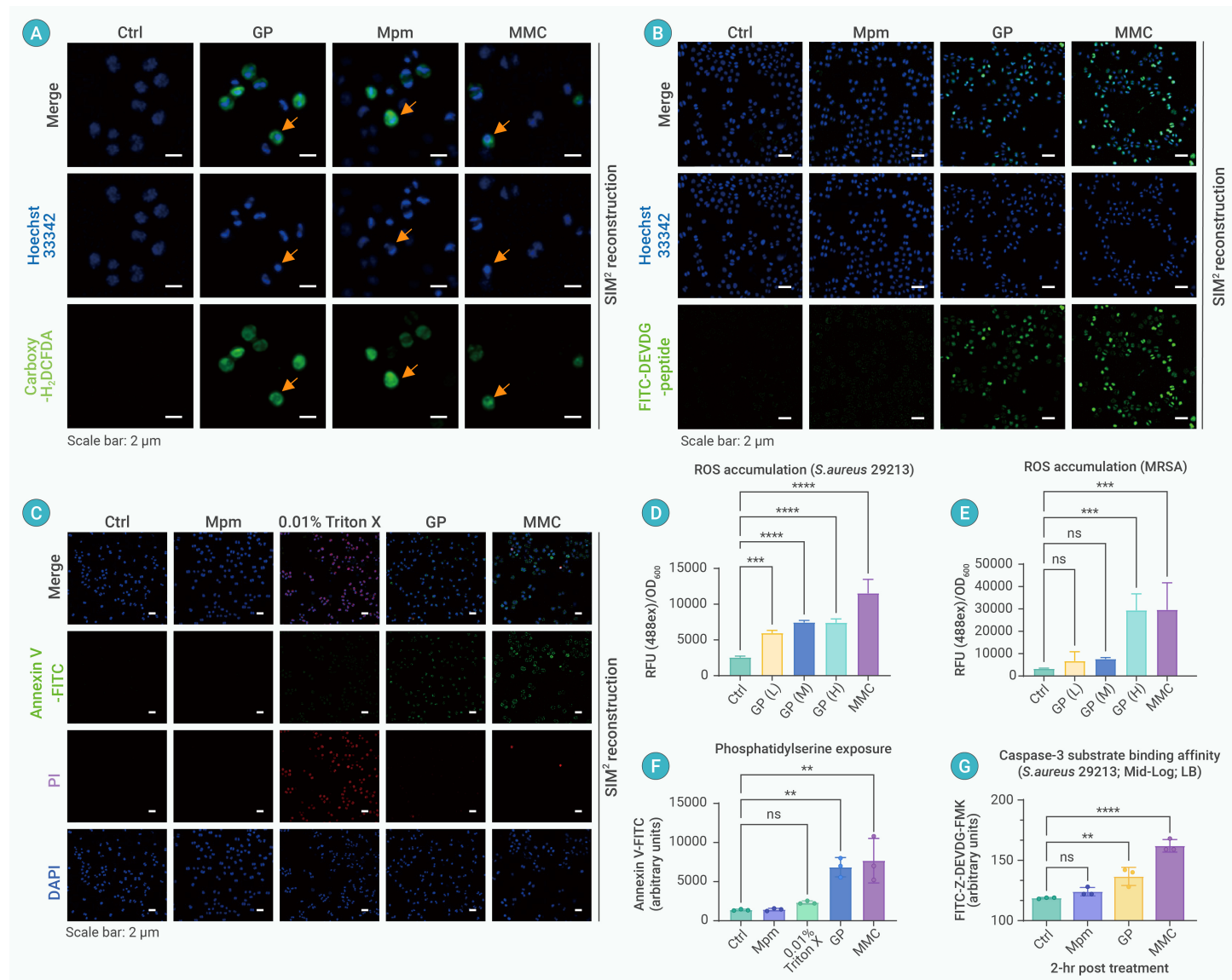


Figure 5. GP induces apoptotic-like cell death of *S. aureus* (A) The reactive oxygen species (ROS) production and chromosomal condensation of *S. aureus*. The arrow-marked part illustrates ROS production accompanied by chromatin condensation. (B) Intracellular activity of caspase-3 like protease in *S. aureus*. (C) Apoptosis in *S. aureus* under different treatments by Annexin V-FITC/PI staining. (D-E) The reactive oxygen species (ROS) assay evaluate the resistance of compound GP in *S. aureus* 29213 and MRSA strain. (F) The semi-quantitative fluorescence results of Annexin V-FITC. (G) The semi-quantitative fluorescence results of caspase-3 like protease. Data are shown as mean $\pm$ SD ($n \geq 3$). Statistical analysis of Lung Index & CFUs was conducted by application of ordinary One-way ANOVA multiple comparison. ns, no significant difference, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Prenylated flavonoids accelerated *S. aureus* fatty acid metabolism

To investigate the mechanism underlying GP-induced ATP hyperconsumption, prompted by observations of membrane depolarization and apoptosis-like phenotypes, targeted fatty acid metabolomics was performed via GC-MS following central carbon metabolomic analysis. As illustrated in Figure 7A, GP treatment led to a significant upregulation of fatty acids cis-5,8,11,14,17-eicosapentaenoic acid, decanoate, cis-11,14,17-eicosatrienoic acid, and linolealaidate in *S. aureus* 29213, which were crucial for membrane stability and signal transduction. Conversely, cis-10-heptadecenoate, elaidate, and stearate were markedly downregulated. Stearate is not only involved in the synthesis of fatty acids, but can also be broken down into acetyl-CoA in mitochondria through the process of fatty acid β -oxidation, and further participates in the tricarboxylic acid cycle and oxidative phosphorylation to provide energy. Downregulated stearate level implied that GP might repair cell damage and improve cell function, resulting in remarkably increased energy demand. Elaidate could induce phospholipid turnover, leading to changes in the physical and chemical properties of cell membranes, and ultimately inducing cell apoptosis. In addition, elaidate mediates Ca^{2+} level disorder through the energy endoplasmic reticulum coupling structure,

inducing energy dysfunction. The downregulation of elaidate in GP-treated ATCC29213 suggested that GP might help preventing the interaction of elaidate with cell membranes and the disturbance of Ca^{2+} levels to ensure cell functionality.

This result supported GP-treatment facilitated metabolomic chaos in fatty acid, which is critical for bacterial membrane normality and functionality.⁴⁰ Accordingly, we assume the candidate drug target of GP has the capability in regulation of the fatty acid metabolism homeostasis.

FarR was identified as the antimicrobial target of GP

To find out the direct drug target interacted with GP, we used the most classical protocol, induction of spontaneous mutant with survival capability under GP treatment since drug target site modification was a common strategy adopted by bacterial cells.^{25,41}

Specifically, we applied serial passage of bacterial cells treated with gradually concentration-enhanced GP to allow spontaneous mutation in whole community. Accordingly, we acquired one isolate with weak GP resistance. This isolate was sequenced using whole genome sequencing to determine genetic mutations associated with the persistent phenotype. Sequencing of genomic DNA revealed mutation in FarR gene, encoding a transcriptional

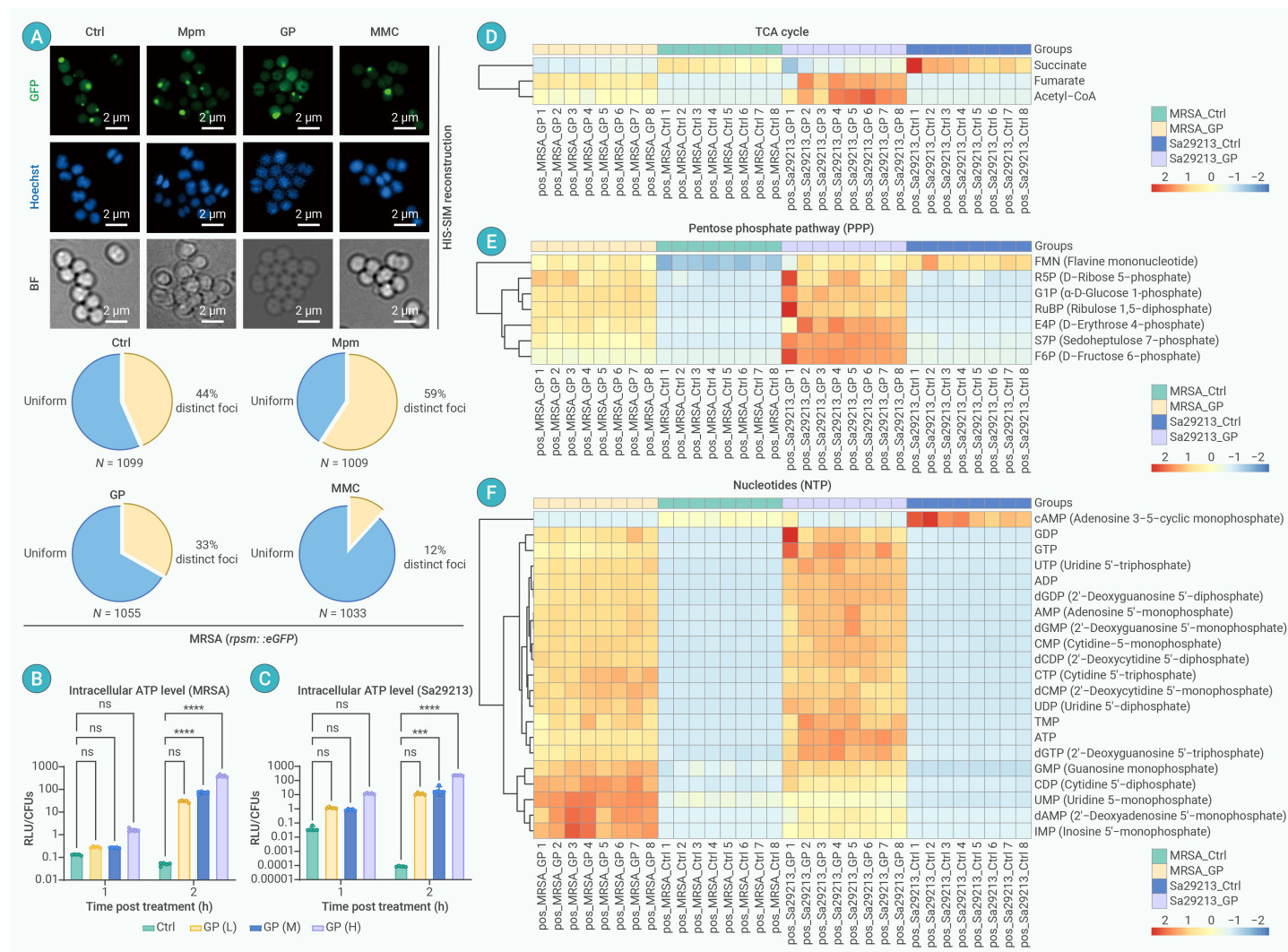


Figure 6. GP causes protein aggregation and energy metabolic shift of *S. aureus* (A) Protein aggregates in *S. aureus* under different treatments using HIS-SIM microscopy. (B-C) Bacterial cellular ATP level measurement. Data are shown as mean $\pm$ SD ($n \geq 3$). Statistical analysis of Lung Index & CFUs was conducted by application of ordinary One-way ANOVA multiple comparison. ns, no significant difference, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. (D-F) Hierarchical clustering heatmap of differential metabolites (2-fold changes; $P < 0.05$) of MRSA & *S. aureus* 29213 (Sa29213) in positive mode. TCA cycle (D); pentose phosphate pathway metabolites (E); nucleotides metabolites (F).

repressor that functions in regulating fatty acid metabolism, was associated with the antibiotic tolerance phenotype.⁴² Interestingly, FarR has been well identified and studied as regulator of bacterial cell fatty acid.^{42,43} Linking to the results above including membrane deterioration, ATP level enhancement, central carbon and fatty acid metabolism elevation, we speculated drug-target interaction of GP-FarR is critical for the magic effects of GP against *S. aureus*.

To confirm that FarR was the target of GP, we evaluated the binding affinity between GP and FarR using bio-layer interferometry (BLI), as depicted in Figure 7B. The analysis revealed a dissociation constant (K_d) of 3.31×10^{-4} M, indicating a measurable interaction. Subsequently, we constructed a pLI50-FarR plasmid to overexpress FarR in *S. aureus*. Notably, the overexpression of FarR markedly enhanced the bacterial resistance to GP, suggesting that FarR influences the sensitivity of *S. aureus* to the compound (Figures 7C-F & S9). These findings corroborated the hypothesis that FarR was a primary target of GP and played a pivotal role in the bacterial response to this compound. Besides, molecular docking studies were conducted to predict potential binding sites of compound GP on FarR. The analysis successfully pinpointed two primary binding sites, situated at residues Tyr69 and Glu151. According to the results, residues Tyr69 and Glu151 could contribute to the binding affinity via hydrogen bonding with the compound (Figure 7G).

Taking together, these results, with the above ones, supported that GP directly interacted with FarR, alongside fatty acid metabolism chaos and

finally leading to bacteria persister cell to programmed cell death.

FarR promotes *S. aureus* persister formation

Since FarR was critical for GP-mediated bacterial cell killing, more than GP alone, we aimed to study if it was a candidate drug target for further study against persister and confirm its role in persister formation. To investigate, the growth curves and metabolic activity before and after FarR overexpression were conducted.

As shown in Figure 8A, FarR overexpression led to a significant alteration in the bacterial growth curve. Notably, the bacterial growth rate was substantially reduced, and the lag phase was markedly prolonged, suggesting that FarR induces a metabolic slowdown or shift in energy usage. In addition, intracellular levels of ROS and ATP in the stationary phase of *S. aureus*, both with and without plasmid-induced FarR expression, were assessed under meropenem (Mpm) stress (Figures 8B-E). In control strains, both ROS and ATP levels increased after meropenem treatment, indicating active metabolic responses. In contrast, comparing to the Ctrl group, FarR-overexpressing strains exhibited a significantly less enhancement in ROS and ATP levels after 2 hours of meropenem exposure, suggesting a metabolic downregulation characteristics of persister formation attributed to FarR protein. Furthermore, Figures 8F-G show a significant increase in protein aggregates in FarR-overexpressing strains. These findings suggest that FarR plays a pivotal role in regulating multiple facets of the persister phenotype in *S. aureus*, including metabolic activity, ROS management, and protein homeostasis. The ability of

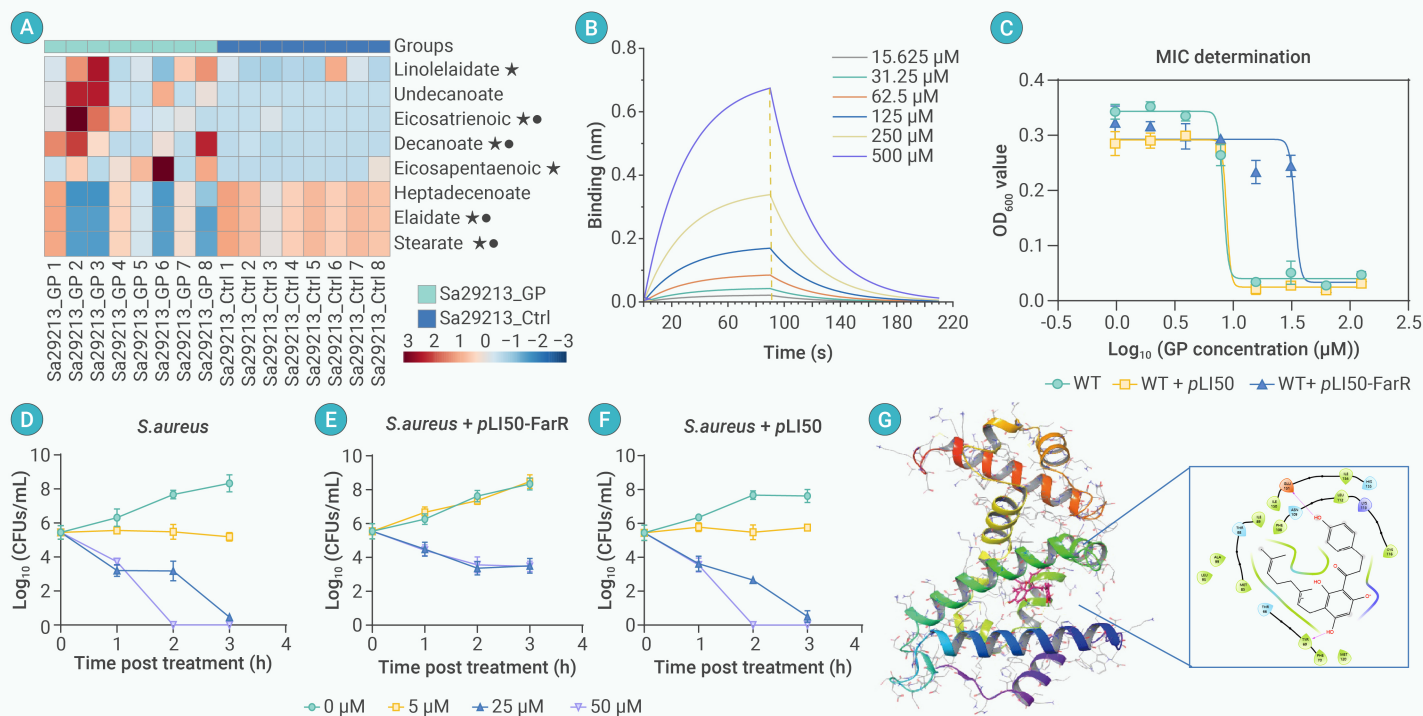


Figure 7. GP directly binds to FarR in *S. aureus* and modulates fatty acid metabolism (A) Heatmaps of differential fatty acids of GP-treated ATCC29213 strains. The fatty acids marked with ★ are closely related to the physiological functions of cell membranes. The fatty acids marked with ● are involved in mitochondrial metabolism. (B) Biolayer interferometry (BLI) analysis evaluate the binding of compound GP to the FarR regulatory protein. (C) The MIC determination of GP in *S. aureus* following the overexpression of the FarR regulatory protein. (D-F) The colony-forming unit (CFU) assay evaluate the resistance of compound GP in *S. aureus* RN4220 wild-type, *S. aureus* RN4220 overexpressing pLI50-FarR and *S. aureus* RN4220 pLI50 overexpression group. Data are shown as mean $\pm$ SD ($n \geq 3$). Statistical analysis of Lung Index & CFUs was conducted by application of ordinary One-way ANOVA multiple comparison. ns, no significant difference, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. (G) The molecular docking analysis between compound GP and the FarR regulatory protein.

FarR to modulate these processes positions it as a critical player in the survival of *S. aureus* under antibiotic stress. Given its central role in persistence, FarR may represent a potential target for therapeutic strategies aimed at reducing the formation of persistent populations and improving the efficacy of antibiotic treatments.

DISCUSSIONS

The data clearly demonstrated that prenylated flavonoids exhibited selective antibacterial activity against Gram-positive bacteria, including MRSA and *S. aureus*, while showing no efficacies against Gram-negative species such as *Pseudomonas aeruginosa* and *Escherichia coli*. This specificity suggested that these flavonoids might help preserving beneficial Gram-negative flora in clinical settings.⁴⁴⁻⁴⁶ MRSA persister cells, which exhibit phenotypic tolerance to antibiotics, pose a significant challenge in the treatment of chronic bacterial infections. The mechanisms of MRSA persister cells including biofilm formation, quorum sensing, and stress response pathways.^{47,48} Biofilm formation significantly diminishes the capacity of host immune system to eliminate infections, thereby escalating the risk of persistent infections.⁴⁹ Notably, GP exhibited bactericidal activity against both actively dividing and dormant MRSA persisters at $< 1 \mu\text{M}$ concentrations. Unlike conventional antibiotics (vancomycin, meropenem, gentamicin), GP eradicated antibiotic-induced persisters by disrupting membrane integrity and generating ROS, which together enhanced cellular lethality under stress conditions.⁵⁰

Additionally, GP inhibited biofilm formation and disrupted pre-formed biofilms, further supporting its clinical relevance, as biofilms serve as a major reservoir of persister cells.⁵¹ The study also highlighted the potential for GP to synergize with established antibiotics, particularly glycopeptides, aminoglycosides, and quinolones. By targeting persister cells that enter a dormant state after antibiotic treatment, GP could enhance the efficacy of these drugs. This synergy likely arises from GP's distinct mechanism of action, which complements the primary mechanisms of conventional antibiotics, such as peptidoglycan synthesis and DNA replication.⁴⁴

The morphological changes and appearance of large planktonic aggregates following GP treatment indicated that autolysis was a significant factor

in the antibacterial action of GP. In *S. aureus*, autolysis is mainly caused by the activation of autolysins, which are enzymes that degrade the peptidoglycan layer of the bacterial cell wall.⁵² The pores and web-like structures within the aggregates resemble biofilms. It is likely that autolysin activity can degrade the cell wall and release the intracellular contents into the extracellular matrix.⁵³ It is worth noting that autolysis may also lead to the production of a secondary extracellular matrix, that is similar in structure to biofilms.⁵⁴ However, it could be a pathological response to the stress and membrane disruption caused by GP. It is likely that the presence of aggregates is a stress response where the bacterial cells are drawn together to minimize the damage from membrane disruption. This aggregation behavior may allow the extracellular matrix created from lysed cells to support the survival of a population of bacteria under unfavorable conditions.

The GP-induced alterations in protein aggregation and metabolism were pivotal in regulating persister cell formation. ATP is crucial for LLPS, both as an energy source for cellular activity, and in improving the solubility of particular proteins, ensuring their correct folding and inhibiting aggregation.⁵⁵ When ATP concentration diminishes, LLPS becomes more favorable, culminating in protein condensation and the subsequent formation of aggregates that might influence stress response and persistence, especially in bacteria like *S. aureus*.⁵⁶ By modifying the energy metabolism and curtailing protein aggregation via LLPS, GP could potentially disrupt the adaptive responses that typically safeguard persister cells.⁵⁶ Such interference might diminish the bacterial capacity to endure antibiotic stress, thereby compromising the generation of dormant, antibiotic-resistant persister cells.⁵⁷ The overall decrease in protein aggregate formation indicated that GP might directly interact vital survival strategies in persister cells, rendering them more vulnerable to treatment. Furthermore, the elevation of energy-related metabolites, such as NADH and NADPH, suggested that GP treatment might promote oxidative phosphorylation and cellular respiration, which could help restoring cellular homeostasis.⁵⁸ This was consistent with studies showing that metabolic shifts play a significant role in bacterial resilience to stress.

Dormant MRSA associated infection, like wound infections, urine tract infection and lung infection are notorious recalcitrance against antibiotic

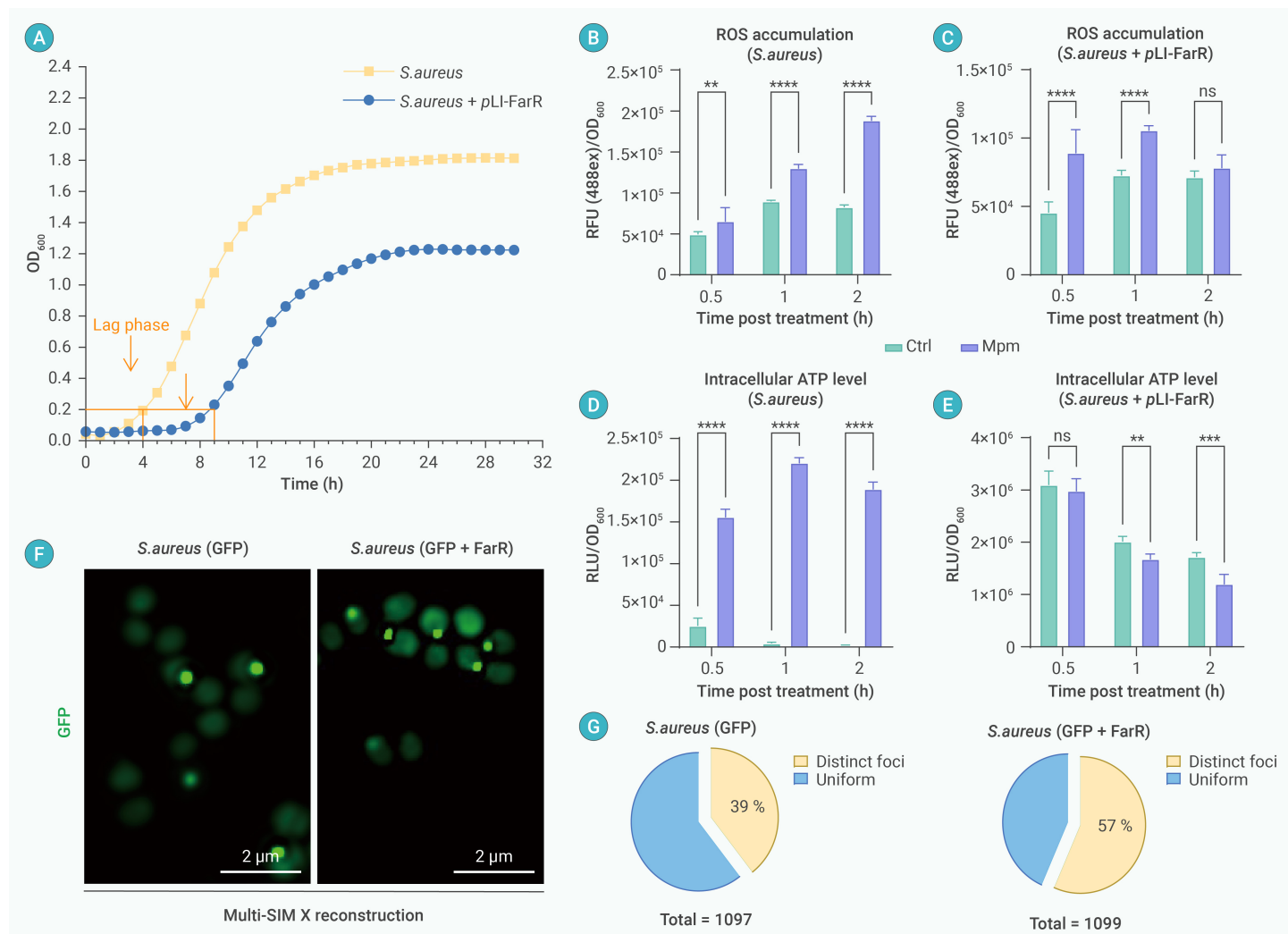


Figure 8. Effect of FarR protein on *S. aureus* persister formation (A) Growth curve of *S. aureus* with or without plasmid expressing FarR protein. (B-E) Intracellular ROS or ATP accumulation of stationary phase *S. aureus* with or without plasmid expressing FarR protein under meropenem (Mpm) stress. (F-G) Representative Figures and semi-quantification of bacterial cell percentage with aggresome (bright foci). *S. aureus* with plasmid expressing GFP at different growth status were introduced with or without FarR protein. Data are shown as mean $\pm$ SD ($n \geq 3$). Statistical analysis was conducted by application of ordinary One-way ANOVA multiple comparison. ns, no significant difference, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

treatment.^{59,60} Killing persisters can efficiently eradicate biofilm infection, which is ineffective for conventional antibiotics via corruption of active metabolic processes.⁶¹ The magic bullets against persisters are rare, nevertheless via apoptosis activation.^{62,63} Our research provided a new approach in persister eradication via apoptosis activation with clinical development potential. Whether bacterial apoptosis in eukaryotic cells include the intrinsic pathway and extrinsic pathway.^{64,65} While the extrinsic pathway is typically induced by developmental signals, the intrinsic pathway is induced primarily by DNA damage and oxidative stress, facilitating the mitochondrial outer membrane permeabilization, caspase-dependent disassembly of cells.⁶⁴ Mitochondrial is believed to originate from endosymbiotic bacteria during evolution.⁶⁶ Besides clinical significance, our study provided a research tool option in study similarity of prokaryotic apoptosis-like cell death and eukaryotic apoptosis. Both might have a common origin and it is likely that intrinsic pathway of apoptosis in the mitochondria in eukaryotic cells is inherited from its evolutionary ancestor of bacterial, and a serial of specialized enzymes dominate the function during evolution, which can be further investigated via GP treatment.

FarR, a TetR-family transcriptional regulator, represses *farE* transcription by binding its promoter under basal conditions.⁶⁷ FarE encodes an efflux pump essential for bacterial tolerance to fatty acids and the regulation of fatty acid metabolism.⁴² Environmental fatty acids or toxic metabolites bind FarR, inducing conformational changes that trigger promoter dissociation and *farE* activation. This enhances efflux of toxic metabolites, maintaining

cellular homeostasis.⁶⁸ Dysregulation of FarR affects efflux pump activity, alters membrane fatty acid composition, impacts antibiotic sensitivity, and compromises membrane integrity. Targeting FarR with compounds like GP offers therapeutic potential by disrupting membrane stability, modulating efflux pump expression, and inducing cell death in resistant bacterial populations. In addition, FarR-overexpression shifts metabolic states toward dormancy, which is characteristic for persister formation.⁶⁹ During this metabolically quiescent lag phase, downregulated biosynthesis reduces antibiotic target availability, consistent with metabolic quiescence in persistence.⁷⁰ Inhibiting FarR or downstream targets may prevent persistence-associated metabolic shifts and protein aggregation, restoring antibiotic susceptibility, particularly relevant for biofilm-associated chronic infections.^{71,72} To the best of our knowledge, this represents the first report linking FarR to bacterial persister formation, LLPS-driven aggresome assembly, and their bactericidal implications. This work reveals fatty acid metabolism as a fundamental component of bacterial persistence, which is distinct from established ATP depletion and central carbon metabolism mechanisms, providing a novel dimension for therapeutic targeting.^{36,37}

This work confirms the bactericidal efficacy of prenylated flavonoids against MRSA persisters and elucidates FarR's critical role in persister formation. Crucially, it establishes a novel therapeutic paradigm targeting bacterial apoptosis to overcome persister-mediated antibiotic relapse. Furthermore, we introduce a flavonoid-based tool for investigating the evolutionary continuum from prokaryotic apoptosis-like death to mitochondria-

mediated eukaryotic apoptosis.

However, limitation of this study is also clear. Correlation between GP-mediated apoptosis and FarR is still unclear, which requires further study in discovery of this mechanism underlied. To our knowledge, beside RecA, the putative prokaryotic caspase-3 protein found in mitomycin-mediated *E. coli* apoptosis, FarR might be another important apoptosis-like cell death regulator found in *S. aureus*. Future research should elucidate how FarR-modulated fatty acid metabolism intersects with RecA-mediated apoptotic pathways.

REFERENCES

- Guo Y., Song G., Sun M., et al. (2020). Prevalence and therapies of antibiotic resistance in *Staphylococcus aureus*. *Front. Cell. Infect. Microbiol.* **10**:107–118. DOI:10.3389/fcimb.2020.00107
- Westgeest A. C., Hanssen J. L., de Boer M. G., et al. (2024). Eradication of community-onset Methicillin-resistant *Staphylococcus aureus* carriage: A narrative review. *Clin. Microbiol.* **31**:173–181. DOI:10.1016/j.cmi.2024.01.003
- Peel T. N., Astbury S., Cheng A. C., et al. (2023). Trial of vancomycin and cefazolin as surgical prophylaxis in arthroplasty. *N. Engl. J. Med.* **389**:1488–1498. DOI:10.1056/NEJMoa2301401
- Garoy E. Y., Gebreab Y. B., Achila O. O., et al. (2019). Methicillin - resistant *Staphylococcus aureus* (MRSA): Prevalence and antimicrobial sensitivity pattern among patients—a multicenter study in asmara, eritrea. *Can. J. Infect. Dis. Med. Microbiol.* DOI:10.1155/2019/8321834.
- Lee A. S., De Lencastre H., Garau J., et al. (2018). Methicillin-resistant *Staphylococcus aureus*. *Nat. Rev. Dis. Primers* **4**:1–23. DOI:10.1038/nrdp.2018.33
- David M. Z., and Daum R. S. (2010). Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. *Clin. Microbiol. Rev.* **23**:616–687. DOI:10.1128/cmr.00081-09
- Hasanpour A. H., Sepidarkish M., Mollalo A., et al. (2023). The global prevalence of methicillin-resistant *Staphylococcus aureus* colonization in residents of elderly care centers: A systematic review and meta-analysis. *Antimicrob. Resist. Infect. Control.* **12**:4. DOI:10.1186/s13756-023-01210-6
- Doyle J., Bachar R., Paul S., et al. (2024). Unmasking a menace: Methamphetamine, mrsa and an elusive abscess leading to pulmonic valve endocarditis. *J. Am. Coll. Cardiol.* **83**:4418–4418. DOI:10.1016/S0735-1097(24)06408-8
- Jones B. E., Ying J., Stevens V., et al. (2020). Empirical anti-MRSA vs standard antibiotic therapy and risk of 30-day mortality in patients hospitalized for pneumonia. *JAMA Intern. Med.* **180**:552–560. DOI:10.1001/jamainternmed.2019.7495
- Bradley C. A. (2018). Disrupting MRSA's persisters. *Nat. Rev. Drug Discov.* **17**:394–394. DOI:10.1038/nrd.2018.84
- Decollogny M., and Rottenberg S. (2024). Persisting cancer cells are different from bacterial persisters. *Trends Cancer* **10**:5. DOI:10.1016/j.trecan.2024.02.002
- Abebe A. A., and Birhanu A. G. (2023). Methicillin resistant *Staphylococcus aureus*: molecular mechanisms underlying drug resistance development and novel strategies to combat. *Infect. Drug Resist* **16**:7641–7662. DOI:10.2147/IDR.S428103
- MacNair C. R., Rutherford S. T., and Tan M. (2024). Alternative therapeutic strategies to treat antibiotic-resistant pathogens. *Nat. Rev. Microbiol.* **22**:262–275. DOI:10.1038/s41579-023-00993-0
- Adefisoye M. A., and Olaniran A. O. (2023). Antimicrobial resistance expansion in pathogens: a review of current mitigation strategies and advances towards innovative therapy. *JAC-AMR* **5**:127. DOI:10.1093/jacamr/dlad127
- Nandhini P., Kumar P., Mickymaray S., et al. (2022). Recent developments in methicillin-resistant *Staphylococcus aureus* (MRSA) treatment: A review. *Antibiotics* **11**:606. DOI:10.3390/antibiotics11050606
- Vasudevan U. M., and Lee E. Y. (2020). Flavonoids, terpenoids, and polyketide antibiotics: Role of glycosylation and biocatalytic tactics in engineering glycosylation. *Biotechnol. Adv.* **41**:107550. DOI:10.1016/j.biotechadv.2020.107550
- Puksasook T., Kimura S., Tadtong S., et al. (2017). Semisynthesis and biological evaluation of prenylated resveratrol derivatives as multi-targeted agents for Alzheimer's disease. *J. Nat. Med.* **71**:665–682. DOI:10.1007/s11418-017-1097-2
- Zheng E. J., Valeri J. A., Andrews I. W., et al. (2024). Discovery of antibiotics that selectively kill metabolically dormant bacteria. *Cell Chem. Biol.* **31**:712–728. e719. DOI:10.1016/j.chembiol.2023.10.026.
- Wong F., Zheng E. J., Valeri J. A., et al. (2024). Discovery of a structural class of antibiotics with explainable deep learning. *Nature* **626**:177–185. DOI:10.1038/s41586-023-06887-8
- Mandakhalikar K. D., Rahmat J. N., Chiong E., et al. (2018). Extraction and quantification of biofilm bacteria: Method optimized for urinary catheters. *Sci. Rep.* **8**:8069. DOI:10.1038/s41598-018-26342-3
- Elgamoudi B. A., and Korolik V. (2023). A guideline for assessment and characterization of bacterial biofilm formation in the presence of inhibitory compounds. *Bio-protoc* **13**:e4866. DOI:10.3390/antibiotics9110836
- Huang X., Fan J., Li L., et al. (2018). Fast, long-term, super-resolution imaging with Hessian structured illumination microscopy. *Nat. Biotechnol.* **36**:451–459. DOI:10.1038/nbt.4115
- Zhao W., Zhao S., Li L., et al. (2022). Sparse deconvolution improves the resolution of live-cell super-resolution fluorescence microscopy. *Nat. Biotechnol.* **40**:606–617. DOI:10.1038/s41587-021-01092-2
- Pu Y., Li Y., Jin X., et al. (2019). ATP-dependent dynamic protein aggregation regulates bacterial dormancy depth critical for antibiotic tolerance. *Mol. Cell* **73**:143–156. DOI:10.1016/j.molcel.2018.10.022
- Le P., Kunold E., Maccs R., et al. (2020). Repurposing human kinase inhibitors to create an antibiotic active against drug-resistant *Staphylococcus aureus*, persisters and biofilms. *Nat. Chem.* **12**:145–158. DOI:10.1038/s41557-019-0378-7
- Zhang N., Shan W., Gao L., et al. (2023). Repurposing the Hedgehog pathway inhibitor, BMS-833923, as a phosphatidylglycerol-selective membrane-disruptive colistin adjuvant against ESKAPE pathogens. *Int. J. Antimicrob. Agents* **62**:106888. DOI:10.1016/j.ijantimicag.2023.106888
- Yuan Z., Wang J., Qu Q., et al. (2023). Celastrol combats methicillin - resistant *Staphylococcus aureus* by targeting 51 - pyrroline - 5 - carboxylate dehydrogenase. *Adv. Sci.* **10**:2302459. DOI:10.1002/adv.202302459
- Ueki H., Wang L., Fukuyama S., et al. (2018). In vivo imaging of the pathophysiological changes and neutrophil dynamics in influenza virus-infected mouse lungs. *PNAS* **115**:E6622–E6629. DOI:10.1073/pnas.1806265115
- Looney M. R., Thornton E. E., Sen D., et al. (2011). Stabilized imaging of immune surveillance in the mouse lung. *Nat. Methods* **8**:91–96. DOI:10.1038/nmeth.1543
- Luo J., Luo J., Sheng Z., et al. (2024). Latest research progress on anti-microbial effects, mechanisms of action, and product developments of dietary flavonoids: A systematic literature review. *Trends Food Sci. Technol.* **156**:104839. DOI:10.1016/j.tifs.2024.104839
- Conlon B. P., Rowe S. E., Gandt A. B., et al. (2016). Persister formation in *Staphylococcus aureus* is associated with ATP depletion. *Nat. Microbiol.* **1**:1–7. DOI:10.1038/nmicrobiol.2016.51
- Dwyer D. J., Camacho D. M., Kohanski M. A., et al. (2012). Antibiotic-induced bacterial cell death exhibits physiological and biochemical hallmarks of apoptosis. *Mol. Cell* **46**:561–572. DOI:10.1016/j.molcel.2012.04.027
- Pacios O., HerreraEspejo S., Armán L., et al. (2024). Mitomycin C as an anti-persister strategy against *Klebsiella pneumoniae*: toxicity and synergy studies. *Antibiotics* **13**:815. DOI:10.3390/antibiotics13090815
- Kwan B. W., Chowdhury N., and Wood T. K. (2015). Combatting bacterial infections by killing persister cells with mitomycin C. *Environ. Microbiol.* **17**:4406–4414. DOI:10.1111/1462-2920.12873
- Li B., Dou S., Yuan J., et al. (2018). Intracellular transport is accelerated in early apoptotic cells. *PNAS* **115**:12118–12123. DOI:10.1073/pnas.1810017115
- Manuse S., Shan Y., Canas S. J., et al. (2021). Bacterial persisters are a stochastically formed subpopulation of low-energy cells. *PLOS Biol.* **19**:e3001194. DOI:10.1371/journal.pbio.3001194
- Jin X., Lee J., Schaefer C., et al. (2021). Membraneless organelles formed by liquid-liquid phase separation increase bacterial fitness. *Sci. Adv.* **7**:eab2929. DOI:10.1126/sciadv.abh2929
- Mo Y., Wang K., Li L., et al. (2023). Quantitative structured illumination microscopy via a physical model-based background filtering algorithm reveals actin dynamics. *Nat. Commun.* **14**:3089. DOI:10.1038/s41467-023-38808-8
- Shan Y., Brown Gandt A., Rowe S. E., et al. (2017). ATP-dependent persister formation in *Escherichia coli*. *MBio* **8**:1. DOI:10.1128/mbio.02267-16
- Zhang Y., and Rock C. O. (2008). Membrane lipid homeostasis in bacteria. *Nat. Rev. Microbiol.* **6**:222–233. DOI:10.1038/nrmicro1839
- Thomas S. S., Kalia N. P., and Pethe K. (2022). Turbidity-based MIC assay and characterization of spontaneous drug resistant mutants in mycobacterium ulcerans. *Methods Protoc.* **2387**:209–217. DOI:10.1007/978-1-0716-1779-3_20
- Alnaseri H., Kuiack R. C., Ferguson K. A., et al. (2019). DNA binding and sensor specificity of FarR, a novel TetR family regulator required for induction of the fatty acid efflux pump FarE in *Staphylococcus aureus*. *J. Bacteriol.* **201**:10.1128/jb.00602-00618. DOI:10.1128/jb.00602-18.
- Iram S. H., and Cronan J. E. (2005). Unexpected functional diversity among FadR fatty acid transcriptional regulatory proteins. *J. Biol. Chem.* **280**:32148–32156. DOI:10.1074/jbc.M504054200
- Wang Z., Zhu Y., Bai L., et al. (2024). A new therapeutic strategy for infectious diseases against intracellular multidrug-resistant bacteria. *J. Control Release* **375**:467–477. DOI:10.1016/j.jconrel.2024.09.028
- Huang J., Zhong X. F., and Gao Y. Z. (2024). New antibiotic against multi-drug resistant bacteria. *Innov. Life* **2**:100057. DOI:10.59717/j.xinn-life.2024.100057
- Wang L., Zhang Y., Xu J., et al. (2023). Listening to enteric bacteria from the perspective of antibiotic alternatives in animal husbandry. *Innov. Life* **1**:100022–100021–100022–100014. DOI:10.59717/j.xinn-life.2023.100022.
- Heo H. Y., Zou G., Baek S., et al. (2024). A methylazanediyol bisacetamide derivative sensitizes *Staphylococcus aureus* persisters to a combination of gentamicin and daptomycin. *Adv. Sci.* **11**:2306112. DOI:10.1002/adv.202306112
- Garrison A. T., Abouelhassan Y., Kallifidas D., et al. (2015). Halogenated phenazines that potentially eradicate biofilms, MRSA persister cells in non - biofilm cultures, and mycobacterium tuberculosis. *Angew. Chem.* **127**:15032–15036. DOI:10.1002/ange.201508155
- Bhattacharya M., and Horswill A. R. (2024). The role of human extracellular matrix

- proteins in defining *Staphylococcus aureus* biofilm infections. *FEMS Microbiol. Rev.* **48**:fuae002. DOI:10.1093/femsre/fuae002
50. Mao C., Xiang Y., Liu X., et al. (2019). Local photothermal/photodynamic synergistic therapy by disrupting bacterial membrane to accelerate reactive oxygen species permeation and protein leakage. *ACS Appl. Mater. Interfaces.* **11**:17902–17914. DOI:10.1021/acsami.9b05787
 51. Antonoplis A., Zang X., Huttner M. A., et al. (2018). A dual-function antibiotic-transporter conjugate exhibits superior activity in sterilizing MRSA biofilms and killing persister cells. *J. Am. Chem. Soc.* **140**:16140–16151. DOI:10.1021/jacs.8b08711
 52. Flores J., Dobihal G. S., Fenton A., et al. (2019). A switch in surface polymer biogenesis triggers growth-phase-dependent and antibiotic-induced bacteriolysis. *ELife* **8**:e44912. DOI:10.7554/eLife.44912
 53. Cheng M., Wu Y., Zeng H., et al. (2024). Asymmetric total synthesis of polycyclic xanthenes and discovery of a walk activator active against MRSA. *Nat. Commun.* **15**:5879. DOI:10.1038/s41467-024-49629-8
 54. Li J., Zhu K., Li C., et al. (2024). Alkaline shock protein 23 (Asp23) - controlled cell wall imbalance promotes membrane vesicle biogenesis in *Staphylococcus aureus*. *J. Extracell. Vesicles* **13**:e12501. DOI:10.1002/jev2.12501
 55. Patel A., Malinowska L., Saha S., et al. (2017). ATP as a biological hydrotrope. *Science* **356**:753–756. DOI:10.1126/science.aaf6846
 56. Schramm F. D., Schroeder K., and Jonas K. (2020). Protein aggregation in bacteria. *FEMS Microbiol. Rev.* **44**:54–72. DOI:10.1093/femsre/fuz026
 57. Saad S., Cereghetti G., Feng Y., et al. (2017). Reversible protein aggregation is a protective mechanism to ensure cell cycle restart after stress. *Nat. Cell Biol.* **19**:1202–1213. DOI:10.1038/ncb3600
 58. Schröder K. (2020). NADPH oxidases: Current aspects and tools. *Redox Biol.* **34**:101512. DOI:10.1016/j.redox.2020.101512
 59. Lebeau D., Ghigo J., and Beloin C. (2014). Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol. Mol. Biol. Rev.* **78**:510–543. DOI:10.1128/mmb.00013-14
 60. Zha Y., Chen C., Jiao Q., et al. (2024). Comprehensive profiling of antibiotic resistance genes in diverse environments and novel function discovery. *Innov. Life* **2**:100054. DOI:10.59717/j.xinn-life.2024.100054
 61. Allison K. R., Brynildsen M. P., and Collins J. J. (2011). Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* **473**:216–220. DOI:10.1038/nature10069
 62. Gollan B., Grabe G., Michaux C., et al. (2019). Bacterial persisters and infection: past, present, and progressing. *Annu. Rev. Microbiol.* **73**:359–385. DOI:10.1146/annurev-micro-020518-115650
 63. Defraigne V., Fauvart M., and Michiels J. (2018). Fighting bacterial persistence: current and emerging anti-persister strategies and therapeutics. *Drug Resist.* **38**:12–26. DOI:10.1016/j.drug.2018.03.002
 64. Kroemer G., Galluzzi L., and Brenner C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiol. Rev.* **87**:99–163. DOI:10.1152/physrev.00013.2006
 65. Nn D. (2004). Cell death: critical control points. *Cell* **116**:205–219. DOI:10.1016/S0092-8674(04)00046-7
 66. Dyall S. D., Brown M. T., and Johnson P. J. (2004). Ancient invasions: from endosymbionts to organelles. *Science* **304**:253–257. DOI:10.1126/science.1094884
 67. Almutairy B. (2024). Extensively and multidrug-resistant bacterial strains: Case studies of antibiotics resistance. *Front. Microbiol.* **15**:1381511. DOI:10.3389/fmicb.2024.1381511
 68. Bonn C. M., Rafiqullah I. M., Crawford J. A., et al. (2023). Repeated emergence of variant TetR family regulator, FarR, and increased resistance to antimicrobial unsaturated fatty acid among clonal complex 5 methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **67**:e00749–00722. DOI:10.1128/aac.00749-22
 69. Pu Y., Li Y., Ma Q., et al. (2017). Dynamic protein aggregation regulates bacterial dormancy depth critical for antibiotic tolerance. *bioRxiv*:233890. DOI:10.1016/j.molcel.2018.10.022
 70. Harms A., Maisonneuve E., and Gerdes K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science* **354**:6318. DOI:10.1126/science.aaf4268
 71. Dwyer D. J., Belenky P. A., Yang J. H., et al. (2014). Antibiotics induce redox-related physiological alterations as part of their lethality. *PNAS* **111**:E2100–E2109. DOI:10.1073/pnas.1401876111
 72. O'Neill J. (2014). Antimicrobial resistance: tackling a crisis for the health and wealth of nations. *Rev. Antimicrob. Resist.* **354**:130949. DOI:10.14894/faruawpsj.51.5_464

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AUTHOR CONTRIBUTIONS

Zhili Sheng and Jincan Luo contributed to formal analysis, investigation, and writing of the original draft. Zifeng Yang and Bao Yang jointly conceived the study, performed formal analysis, and critically edited the manuscript. Other authors conducted formal analysis. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

All animal experiments were conducted according to the protocol approved by the Animal Welfare and Research Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (20240039).

DATA AND CODE AVAILABILITY

All data presented in this study are available from the corresponding author upon reasonable request. This study did not generate novel custom code. Supplemental information necessary for data reanalysis is also available from the corresponding author upon request.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-life.2025.100154>