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Fingolimod as a potent anti-*Staphylococcus aureus*: pH-dependent cell envelope damage and eradication of biofilms/*persisters*

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Abstract

Background The urgent need for new antibacterial drugs has driven interest in repurposing therapies to combat Gram-positive biofilms and *persisters*. Fingolimod, an Food and Drug Administration (FDA)-approved drug for multiple sclerosis, shows bactericidal activity, particularly against Methicillin-resistant *Staphylococcus aureus* (MRSA) and biofilm-related infections. With a well-documented safety profile and strong translational potential, it aligns with World Health Organization's goals for antimicrobial repurposing. However, the action mode and mechanism of Fingolimod against gram-positive bacteria remain elusive.

Methods This study utilized clinical *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecalis* (*E. faecalis*), *Streptococcus agalactiae* (*S. agalactiae*). And their susceptibility to Fingolimod and other antibiotics was tested via Minimum Inhibitory Concentration (MIC) assays. Biofilm inhibition and hemolytic activity were evaluated using crystal violet staining, Confocal Laser Scanning Microscopy (CLSM), and hemolysis assays, respectively, while the effect of phospholipids on Fingolimod efficacy was assessed with checkerboard assays. Membrane permeability and integrity were measured using SYTOX green staining and transmission electron microscopy. Whole-genome sequencing was performed on Fingolimod-resistant *S. aureus* isolates to identify Single Nucleotide Polymorphisms (SNPs) linked to resistance.

Results Our data indicated that Fingolimod exerted bactericidal activity against a wide spectrum of gram-positive bacteria, including *S. aureus*, *E. faecalis*, *S. agalactiae*. Moreover, Fingolimod could significantly eliminate the *persisters*, inhibit biofilm formation and eradicate in-vitro mature biofilms of *S. aureus*. The mechanism by which Fingolimod rapidly eradicated *S. aureus* involved a pH-dependent disruption of bacterial cell permeability and envelope integrity. Concomitantly, exogenous supplementation of phospholipids in the culture medium resulted in a dose-dependent increase in the MIC of Fingolimod. Specifically, the addition of 64 μg/mL of cardiolipin (CL) and phosphatidylethanolamine (PE) completely nullified the bactericidal activity of Fingolimod at a concentration of 4 times the MIC. After four months of Fingolimod exposure, the MIC values of *S. aureus* showed a slight increase, indicating that it is not prone to developing drug resistance.

Conclusion Fingolimod exhibits bactericidal activity against diverse gram-positive bacteria, with remarkable effects on *S. aureus* (including MRSA), disrupting bacterial cell structural integrity in a pH-dependent way and eradicating biofilms and *persisters* of *S. aureus*.

Keywords Fingolimod, *Staphylococcus aureus*, Phospholipids

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Introduction

Staphylococcus aureus has been widely regarded as a common cause of hospital and community-acquired infection. *S. aureus* can cause serious invasive and life-threatening infections, such as endocarditis, osteomyelitis, necrotic pneumonia, sepsis, septic arthritis et al. [1, 2]. With the rapid spread of MRSA and the widespread use of last-line agents such as vancomycin, linezolid, and daptomycin, the emergence of multi-drug resistant *S. aureus* has been increasingly reported and gradually posed a threat for the clinical outcome of *S. aureus* infection [3, 4]. Moreover, antibiotic resistance of *S. aureus* against several new anti-MRSA drugs, such as dalbavancin, telavancin, oritavancin, and tigecycline, also emerged shortly after their introduction [5]. Bacteria biofilm is a complex structure of extracellular polymeric and can protect the cells against antibiotics and hostile conditions, and moreover, the sub-population of the biofilm-embedded bacteria exists in the form of dormant cells with metabolically inactive to antibiotic-tolerant phenotype. A high frequency of clinical *S. aureus* isolates can form the biofilm. Biofilm formation and *persisters* pose tremendous challenges for clinicians in treating *S. aureus* infections [6–9]. Therefore, it is urgent to discover the novel anti-*S. aureus* agents which can overcome drug resistance, kill the *persisters* and eradicate the biofilm.

A multitude of bacterial vital biochemical processes, such as selective permeability, nutrients transportation and aerobic respiration, takes place on the cell membrane. The bacterial membrane plays an essential role in the survival and growth, and can be regarded as an ideal biological target to discover novel anti-microbial agents [10]. Several commonly used antibiotics, such as daptomycin, polymyxin, and bedaquiline, play their bactericidal activity by disrupting the integrity of bacterial membranes [11–13].

Here, Fingolimod shows bactericidal activity against a wide range of gram-positive bacteria. Among them, particularly for *S. aureus*, including MRSA, Fingolimod demonstrates remarkable effects. Notably, Fingolimod disrupts the structural integrity of bacterial cells in a pH-dependent manner and eradicates biofilms and *persisters* against *S. aureus*. Through in-depth analysis of these phenomena, this study further explores and offers a new mechanism of action for Fingolimod in the treatment of *S. aureus* infections.

Materials and methods

Bacterial strains, antibiotics, and chemicals

The strains used in this study included *S. aureus* SA113 and *Escherichia coli* (*E. coli*) ATCC 25922 from the American Type Culture Collection, as well as clinical isolates of *S. aureus*, *E. faecalis*, and *S. agalactiae* from Shenzhen Nanshan People's Hospital. All strains were grown in Tryptic Soya Broth (TSB), Luria–Bertani medium (LB) or Mueller–Hinton broth (MHB) (OXOID, Basingstoke, UK) at 37℃. The Oxacillin (MB5519-1, Meilunbio, Dalian, China), Vancomycin (MB1260-1, Meilunbio, Dalian, China), Linezolid (MB1469, Meilunbio, Dalian, China) and Fingolimod (CAS No. 162359–56-0, MCE, Shanghai, China) were purchased. The dimethylsulfoxide (DMSO) was used to dissolve Fingolimod and the highest DMSO concentration in the incubation medium was 0.5%, which was not affecting the bacterial growth and biofilm formation [16].

MIC and growth curve

MIC was determined by broth microdilution according to CLSI guidelines using the MHB. The representative MIC for each bacterial species was determined by selecting the value that corresponded to the largest number of tested strains. Bacteria were grown overnight and adjusted to 5.0×10^5 CFU/mL mixed with varying concentrations of test antibiotics in 96-well microtiter plates and incubated at 37 °C for 20–24 h. The bacteria strains were diluted 1:200 in TSB with or without drugs and grown for 24 h shaking at 37 °C with 220 rpm, the OD_{600} was detected at 1 h intervals by Bioscreen C (Turku, Finland). Bacterial growth curves in TSB without Fingolimod were used as an untreated control.

Time-kill curve detecting persistence.

A time-kill curve study was carried out to measure the *persisters* of *S. aureus* to varying antibiotics [17]. Overnight SA113 and CHS350 were diluted to a concentration of 10^8 CFU/mL and incubated with 4× MIC or 10 × MIC drugs including Fingolimod, vancomycin, cefazolin, and linezolid. Following a set exposure period to the antibiotic, spin the treated microbial culture at 5000 rpm for 5 min to form a bacterial pellet. Decant the supernatant with antibiotic. Resuspend pellet in sterile, antibiotic-free 0.9% saline. Repeat the centrifugation and resuspension steps 3 times to remove antibiotic. Samples were diluted in 0.9% saline and spread on TSB agar plates, and the CFU was counted at indicated time points. Then, continue to count colonies up to 5 days, as bacteria recovering from antibiotic treatment may have a significantly delayed growth. Multiple comparisons among varying antibiotic groups mean differences were performed using Dunnett's test. All of the experiments were repeated in triplicate.

The biofilm assays

The inhibition and eradication of biofilm by Fingolimod was performed by crystal violet assay and CLSM according to a previously reported method [18]. Simply say, the bacteria isolates were incubated in TSBG (TSB with 0.5%

glucose) individually into 96-well polystyrene microtiter plates or a cell culture dish inlaying a glass coverslip (World Precision Instruments, USA) containing various concentrations of Fingolimod for indicated times. In the experiment of inhibiting biofilm formation, *S. aureus* was subjected to co-culture with Fingolimod at concentrations of 1/8, 1/4, and 1/2 ×MIC for 24 h. Subsequently, it was washed with PBS 3 times. Then either stained with crystal violet and the absorbance of the stained sample was measured at 570 nm, or it was stained with LIVE/DEAD reagents (1 μM SYTO9 and 1 μM propidium iodide [PI]; Thermo Fisher Scientific, Houston, TX) for use in laser scanning confocal microscopy. Then, images were acquired using a Confocal Laser Scanning Microscope (FV3000, OLYMPUS, Japan) with a 60 ×oil immersion objective. In contrast, for the experiment of eradicating biofilm, 1, 2, 4, 8, 16, and 32 ×MIC of Fingolimod were added to the pre-formed mature biofilms that had been grown for 24 h. All of the experiments were repeated at least three times.

Hemolytic activity assay

The hemolytic activity of *S. aureus* impacted by Fingolimod was performed as previously described [19]. *S. aureus* strains were inoculated with diverse concentrations of Fingolimod at 37 °C for 24 h. The supernatant was harvested by centrifugation, filter sterilized through a 0.22 μm filter (Millipore), and mixed with 1% rabbit erythrocytes (SBJ-RBC-RAB003, Sbjbio, China) at a volume ratio of 1:1. Then, the mixture was incubated at 37 °C for 30 min. The supernatant was harvested by centrifugation and measured the OD_{550}. The 0.1% Triton X-100 and saline were served as the positive control of 100% and the negative control of 0% hemolysis. All of the experiments were repeated in triplicate.

Phospholipids impair the antibacterial activity of Figolimod

Fingolimod's antibacterial efficacy was investigated using a modified version of previously published methods [20]. Sigma-Aldrich provided cardiolipin (CL), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). The phospholipids components were individually solubilized as follows: CL in methanol, PE in ethanol, and PG in dimethyl sulfoxide (DMSO), based on their respective solubility properties. The terminal concentrations of all solvents (methanol, ethanol, DMSO) introduced via lipid solutions did not exceed 0.5% in any experimental condition. Using a checkerboard assay, we investigated the impact of phospholipid concentration (0 to 128 g/mL) on Fingolimod's antibacterial activity against SA113 and CHS350.

Permeability and integrity of cell membrane

The cell membrane permeability of bacteria and mammalian cell were determined according to the previous report [21]. The SYTOX green penetrates into the cell and binds to intracellular DNA when the membrane is damaged. The exponentially growing *S. aureus* SA113 and USA300 were centrifugated and resuspended in 0.9% NaCl, stained for 30 min by SYTOX green (1 μM), then treated with 0.9% NaCl (negative control), 1% Triton X-100 (positive control), 2× MIC and 4× MIC of Fingolimod, daptomycin and linezolid only for *S. aureus* for 30 min. The fluorescence was monitored for 20 min at 37 °C using a BioTek multifunctional microplate reader under the conditions of excitation wavelength of 490 nm and emission wavelength of 520 nm, respectively. All of the experiments were repeated in triplicate.

The cell membrane integrity of *S. aureus* was observed by transmission electron microscopy. The exponentially growing *S. aureus* SA113 cells were treated with 4× MIC Fingolimod for 30 min. Cells were collected, washed three times with PBS (pH = 7.4), and fixed with 2.5% paraformaldehyde/PBS solution. The cells were washed with 0.1 M phosphoric, fixed with acid 1% osmic acid for 2 h at 4 ℃, dehydrated, and embedded in paraffin. Then the samples were sliced into 70 nm sections, stained with 3% uranium acetate-citric acid, and imaged by a transmission electron microscope (HT7800, HITACHI, Japan).

In vitroinduction and the whole-genome sequencing of Fingolimod resistance *S. aureus* isolates

S. aureus isolates (CH101 and SA113) were re-inoculated with 0.5x, 1x, 2x, and 4× of Fingolimod MIC at a dilution ratio of 1:100 every day for 40 and 44 consecutive days until the MIC changed. The individual derivative clone was picked and isolated in TSB plates. The chromosomal DNA of wild-type and Fingolimod-resistant strains of CH101 and SA113 was extracted using the MiniBEST Bacteria Genomic DNA Extraction Kit Ver.3.0 (Takara Biotechnology, Dalian, China) for whole-genome sequencing (Novogene, Beijing, China). The genome of *S. aureus* NCTC 8325 (NCBI Reference Sequence: NC_007795.1) was used as the standard reference to map the sequencing files. Using the MUMmer comparison software, the sequence of resistant strain was serially compared with the non-drug-induced, normally passaged wild-type strain and screened for SNPs according to our previous reports [20].

Proteomic screening of Fingolimod's bacterial targets

Exponentially growing *S. aureus* cells were adjusted to $OD_{600} \approx 0.5$, washed three times with PBS, and centrifuged at 6000 g. Bacterial cells were then incubated

with Fingolimod at 4× MIC for 60 min at 37 °C. The cells were disrupted with 0.1 mm glass beads through high-speed vortexing. The lysate was treated with RNase (0.5 mg/mL) and DNase (0.75 mg/mL) on ice for 1 h, followed by the addition of 5 mM TBP and alkylation with 15 mM iodoacetamide. Total protein concentration was measured, and 100 μg of total protein was digested with trypsin and labeled with TMT 10-plex reagents. Proteomic analysis was conducted using a Q Exactive Plus mass spectrometer (Thermo Scientific), followed by data analysis.

Statistical analysis

The data were analyzed GraphPad Prism 9 and P values < 0.05 were considered statistically significant.

Results

Bactericidal activity of Fingolimod against the common Gram-positive pathogen

The MIC distribution of Fingolimod against the common Gram-positive pathogen clinical isolates from China was determined by broth microdilution. Fingolimod demonstrated potent bactericidal activity against Gram-positive pathogens, with MICs of 6.25 μg/mL (*S. aureus*), 12.5 μg/mL (*E. faecalis*), and 3.125 μg/mL (*S. agalactiae*) (Table 1).

Subsequently, a time-kill assay was conducted on Fingolimod. The assay included Fingolimod alone and in combination with vancomycin, cefazolin, and linezolid. Two strains of *S. aureus* were used: Methicillin-Sensitive *Staphylococcus aureus* (MSSA) SA113 and MRSA CHS350. The results demonstrated the bactericidal activity of Fingolimod against *S.aureus persisters*. When used as monotherapy, Fingolimod outperformed cefazolin (only used against MSSA SA113), vancomycin (only used against MRSA CHS350), and linezolid (Fig. 1). Moreover, the combination of Fingolimod with either vancomycin or linezolid exhibited a more potent bactericidal effect against MRSA CHS350 compared to their respective monotherapies (Fig. 1B).

The inhibition and eradication of *S. aureus* biofilm formation by Fingolimod

The inhibition of Fingolimod at subinhibitory concentrations of MIC on the biofilm formation of *S. aureus* was investigated in six biofilm-positive *S. aureus* clinical isolates that were previously reported [22], including MSSA strains of SE13, SE16, SA113, YUSA10 and MRSA strains of CHS350, CHS655. The biofilm formation of six *S. aureus* strains was significantly inhibited by 1/2 ×MIC Fingolimod (1.56 μg/mL) (Fig. 2A). This finding was further observed and confirmed by laser scanning confocal microscopy using *S. aureus* SA113 (Fig. 2B). Furthermore, the eradication activity of Fingolimod against *S. aureus* SA113 mature biofilm was evaluated by crystal violet staining and laser scanning confocal microscopy, indicating that Fingolimod monotherapy at concentrations equal to or greater than 4 times the MIC could more strongly eradicate the mature biofilm when compared with vancomycin, linezolid, and daptomycin, which are the drugs for clinical MRSA treatment (Fig. 2C). The results of eradicating the mature biofilm were also verified using confocal microscopy.

Disruption of S. aureus cell envelope and membrane permeability by Fingolimod

The cell membrane permeability of two *S. aureus* strains stained with SYTOX green was assessed using Fingolimod, daptomycin, and linezolid. The 1% TritonX-100 served as a positive control for increased *S. aureus* membrane permeability. Our data indicated Fingolimod effectively damaged the *S. aureus* membrane and enhanced the permeability of *S. aureus* (Fig. 3A and B). Notably, daptomycin, which targets the cell membrane, significantly increased the membrane permeability of *S. aureus*, while linezolid, which does not target the cell membrane, showed no such change. Electron microscopy of *S. aureus* SA113, grown planktonically to the mid-log phase and treated with Fingolimod for 30 min, revealed cell envelope disruption (Fig. 3C and D). These findings suggest that Fingolimod's bactericidal effect on *S. aureus* is likely due to rapidly increasing its permeability and disrupting cell envelope integrity.

A previous study proved that the chemical structure of Fingolimod contained a side-chain -NH_2, which could be

Table 1 The MIC values of Fingolimod in different bacteria

Bacteria species	No	Fingolimod MIC distribution (μg/mL)					MIC (μg/mL)
		1.56	3.12	6.25	12.5	> 200	
S. aureus	73	1	42	29	1	0	3.12
E. faecalis	76	0	6	61	9	0	6.25
S. agalactiae	69	40	29	0	0	0	1.56

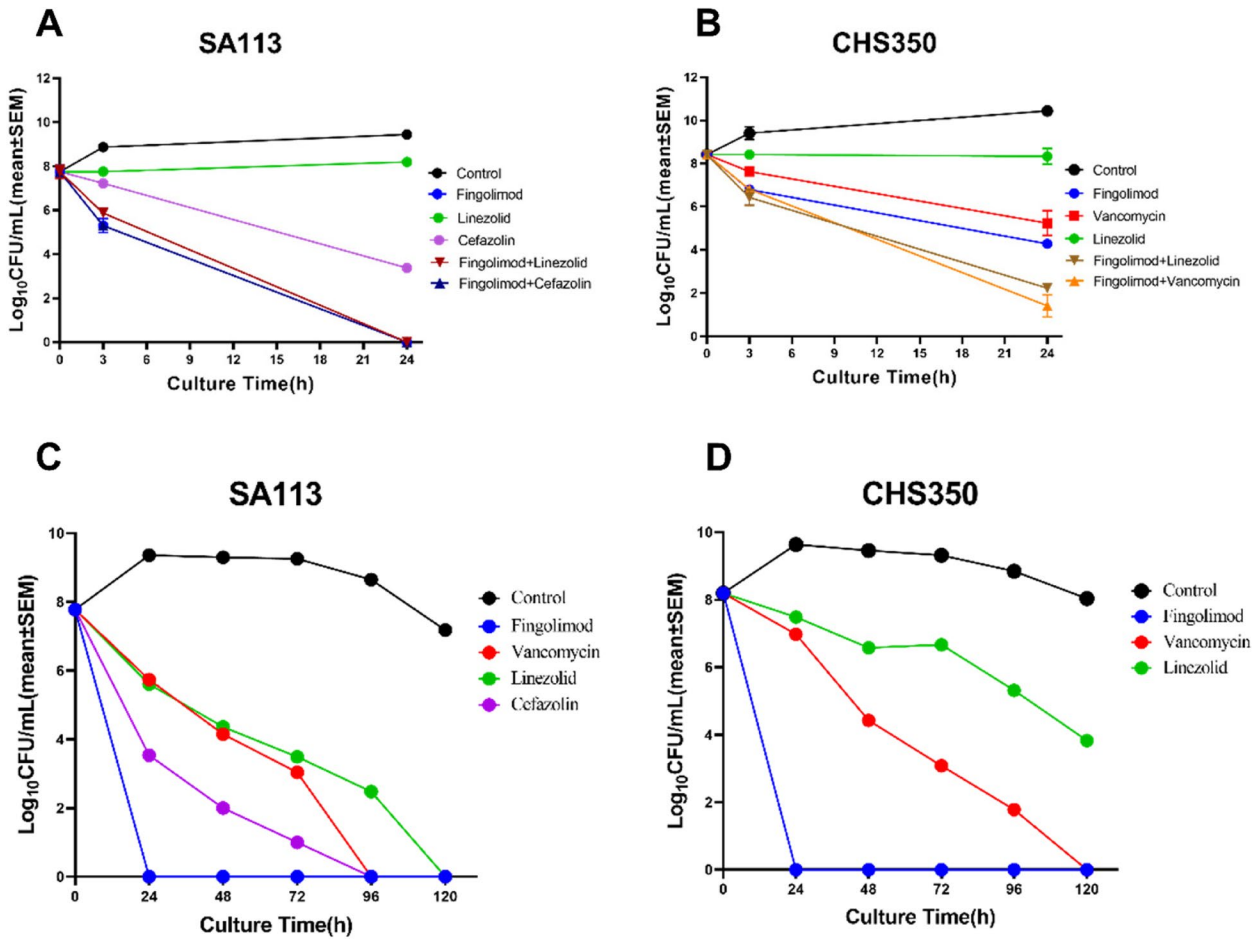

Fig. 1 The bactericidal activity of Fingolimod against planktonic cells and *persisters* of *S. aureus*. Overnight cultures of MSSA SA113 (**A**) and MRSA CHS350 (**B**) were adjusted to 10^8 CFU/mL and treated with 4 × MIC concentrations (Fingolimod: 12.5 μg/mL; vancomycin [MRSA only]: 2 μg/mL; cefazolin [MSSA only]: 2 μg/mL; linezolid: 2 μg/mL). Planktonic cell viability was quantified by CFU counts at 0, 3, and 24 h. And the MSSA SA113 (**C**) and MRSA CHS350 (**D**) incubated with 10 × MIC concentrations (Fingolimod: 32 μg/mL; vancomycin [MRSA only]: 5 μg/mL; cefazolin [MSSA only]: 5 μg/mL; linezolid:5 μg/mL). Planktonic cell viability was quantified by CFU counts at 24, 48, 72, 96 and 120 h for *persisters*. The negative control was DMSO. The data presented was the average of three independent experiments (mean ± SD)

protonated to present in the form of $-NH^{3+}$ under the slightly acidic condition, as found in the normal airway surface liquid [23]. Therefore, whether the protonation of $-NH_2$ was required for the bactericidal effects of Fingolimod was validated by assaying the growth curves of bacteria under various pH conditions. Our data showed that Fingolimod completely suppressed the planktonic growth of bacteria at a concentration of 6.25 μg/mL under pH 6 but not under pH 8, suggesting the protonation of the $-NH^2$ might impact the antibacterial effect of Fingolimod (Fig. 4).

Fingolimod's impact on cell membrane probably via *phospholipids*

In order to identify the potential target site of Fingolimod, the Fingolimod-induced resistant *S. aureus* clones were selected by in vitro serial passaging under the pressure of Fingolimod. The MIC value of SA113 and CHS101 were elevated from 3.125 to 6.25 μg/mL after 40 or 45 passages (Fig. 5). A comparison of whole-genome sequencing between the Fingolimod parental isolates and Fingolimod-induced *S. aureus* clones showed the four coding genetic mutations in the acyl carrier protein synthase (AcpS), inorganic phosphate transport regulatory protein (PhoP and PhoU2) respectively(Supplementary Table1). Moreover, this study compared the proteomes of Fingolimod-treated and DMSO-treated bacteria. The results identified 147 differentially expressed proteins with over two-fold changes, 76 upregulated and 71 downregulated (Fig. 6A). GO analysis indicated that these proteins were primarily associated with redox functions, as well as cell membrane composition and integrity (Fig. 6B). Additionally, ClueGO analysis identified 42 proteins

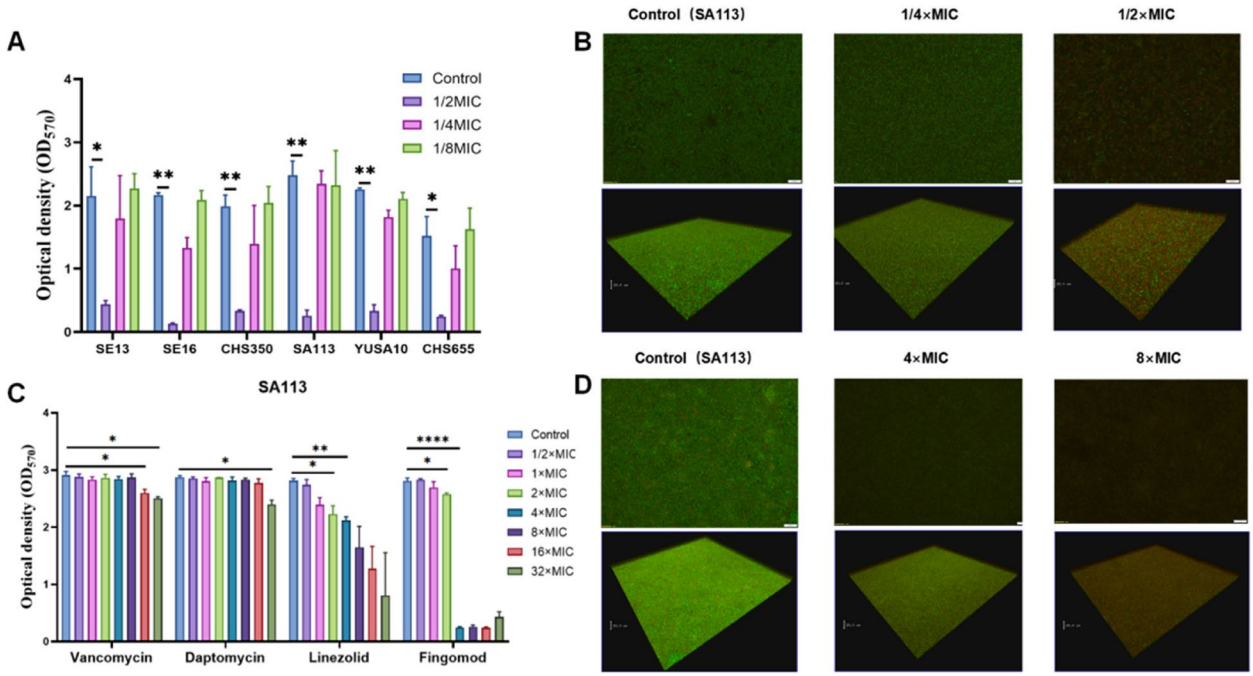

Fig. 2 The inhibition and eradication *S. aureus* biofilm of Fingolimod. The inhibition of *S. aureus* biofilm by Fingolimod was evaluated using (**A**) crystal violet staining or (**B**) laser confocal imaging. The *S. aureus* was co-cultured with different concentrations 1/8, 1/4, and 1/2 × MIC of Fingolimod for 24 h, then washed with PBS for 3 times, stained with crystal violet or treated with syto-9 green fluorescent dye and PI red fluorescent dye for laser scanning confocal microscopy. In contrast, for the experiment assessing biofilm eradication, evaluated by (**C**) crystal violet staining or (**D**) laser confocal imaging,1, 2, 4, 8, 16, and 32 × MIC of Fingolimod were added to the pre-formed mature biofilms that had been grown for 24 h. The negative control was DMSO. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Significant differences compared to the vehicle control group are indicated as *, $P < 0.05$; **, $P < 0.001$. Results represent the mean ± SD of three independent experiments

involved in membrane composition**.** The localization of proteins within *S. aureus* was mapped, and the enriched cell component proteins were visualized (Fig S2). Collectively, these findings suggest that Fingolimod may interfere with the cell membrane by affecting phospholipids.

We evaluated the influence of phospholipid components on the antibacterial and bactericidal activity of Fingolimod using checkerboard assays. This assay employed three common bacterial membrane phospholipids, including PE, PG and CL. After 20 h, the MICs of Fingolimod were measured against clinically isolated strains of SA113 and CH350. The MIC of Fingolimod increased with phospholipid concentration, reaching a 16-fold increase with CL, PG and PE for both isolates (Fig. 7 A and B). Among these phospholipids, CL has a particularly strong influence on increasing the MIC compared to the other two. The results of the time-kill assay after 24 h showed that the addition of 64 μg/mL of CL and PE eliminated the bactericidal activity of Fingolimod at a concentration of 4 times the MIC. However, the addition of PG had no effect on Fingolimod, and there even seemed to be a tendency of enhancement in *S. aureus* SA113 (Fig. 7 C and D).

Discussion

The presence of *persisters* and biofilm formation is closely linked to the recalcitrance of chronic infections and often results in a bad clinical outcome that is associated with significant mortality [24–27]. The conventional antibiotic treatment is frequently difficult to eradicate chronic infections caused by *S. aureus*, in particular MRSA [28]. Previous studies have demonstrated the various physiological uses of survival bacteria cells require the participation of intact membranes. Several reports supported the bacteria membrane is a promising potential target to counter the *persisters* and biofilm for the development of new drugs. To shorten research time and costs, the drug repurposing targeting bacteria membrane by screening libraries of FDA-approved drugs could streamline the need of pharmacokinetic and toxicity [29, 30]. In the present study, our data indicated that Fingolimod can efficiently inhibit the planktonic growth of *S. aureus* with the MIC range from 1.56 μg/mL to 6.25 μg/mL. Moreover, Fingolimod can eradicate the *persisters* and mature biofilms. Time-killing assay demonstrated the stronger bactericidal activity of Fingolimod when compared with vancomycin or linezolid. However, the influence of Fingolimod on the

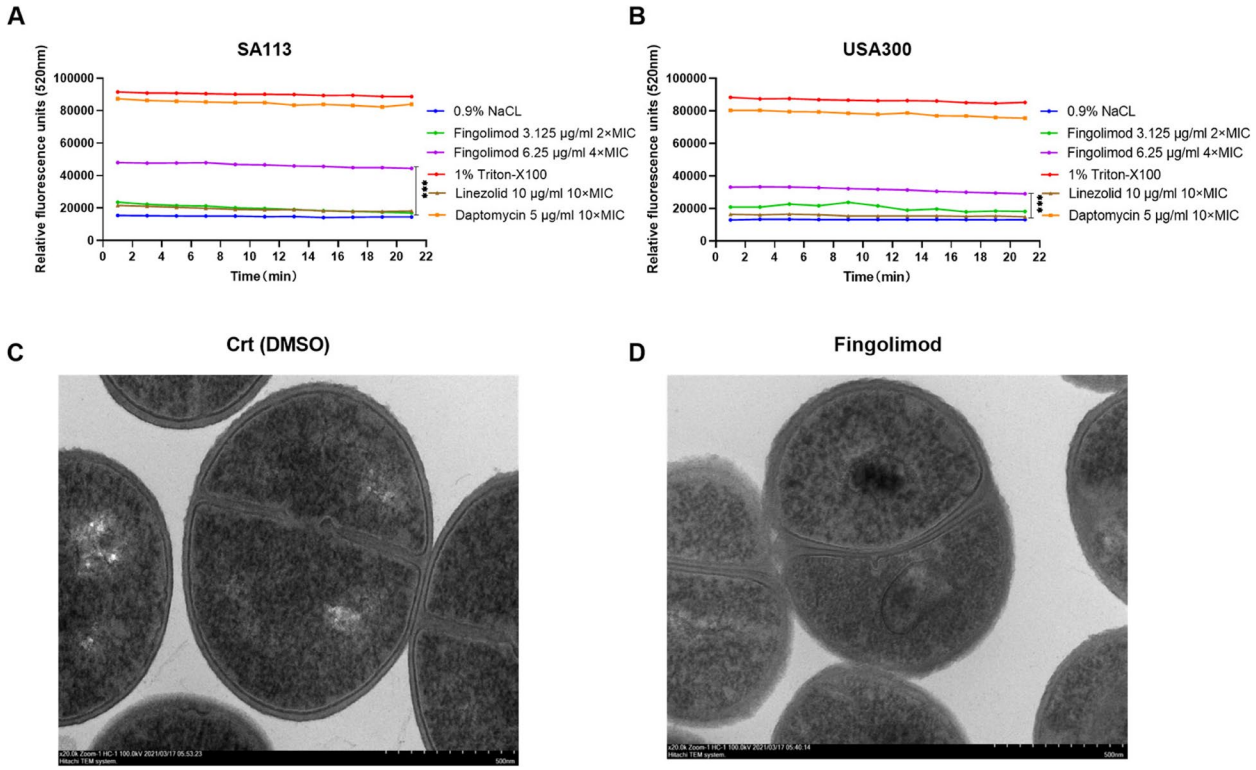

Fig. 3 Fingolimod-induced increase in *S. aureus* cell membrane permeability and disruption of cell integrity. The cell membrane permeability of the MSSA SA113 (**A**), and MRSA CHS350 (**B**) were stained for 30 min by SYTOX green (1 μM) and treated with 0.9% NaCL (negative control), 1% Triton X-100 (positive control), 2× MIC and 4× MIC of Fingolimod for 30 min. Then, BioTek multifunctional microplate reader was used to monitor for 20 min under the conditions of excitation wavelength of 490 nm and emission wavelength of 520 nm. *S. aureus* SA113 cells in the log phase of growth were treated with DMSO (**C**) and 4× MIC Fingolimod (**D**) for 30 min. Cells were collected, washed three times with PBS (pH = 7.4), and fixed with 2.5% paraformaldehyde/PBS solution. The cell disruption were observed by transmission electron microscopy. Compared with the vehicle control group, ***, $P < 0.0001$ (t-test). The data presented was the average of three independent experiments (mean ± SD)

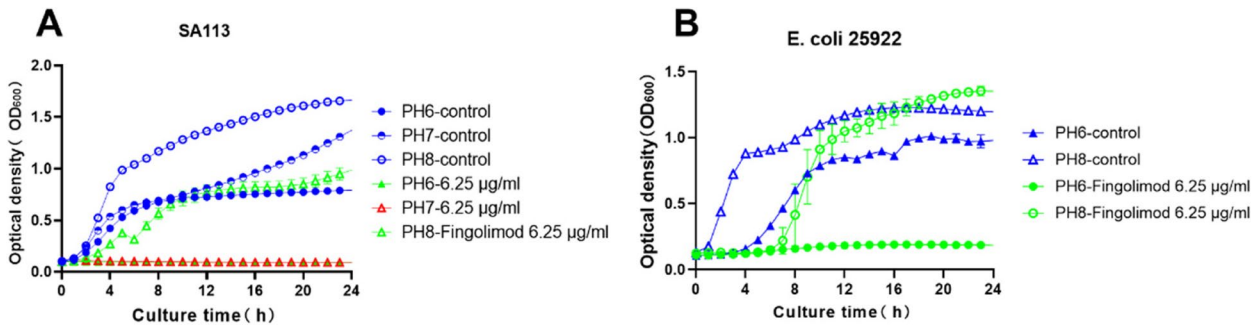

Fig. 4 The protonation of NH_2 is important for the antibacterial effect of Fingolimod. The overnight incubation *S. aureus* SA113were grown in TSB (**A**) and *E. coli* ATCC 25922 were grown in LB (**B**) (pH = 6 and pH = 8) containing Fingolimod (0 or 6.25 μg/mL). The inhibition of the bacterial growth by Fingolimod was detected by measuring the OD_{600} every hour until 24 h in the bacteria automatic growth curve instrument

hemolytic activity of *S. aureus* was also analyzed in 5 clinical *S. aureus* strains using the rabbit erythrocytes, indicating no significant impact on the hemolytic activity by Fingolimod exposure. This effectiveness provides clues that Fingolimod had the potential to provide a novel choice for the antimicrobial treatment of chronic infections of *S. aureus*.

Fingolimod has been approved as the first-line drug for the treatment of the relapsing forms of multiple sclerosis. The clinical application practices have demonstrated

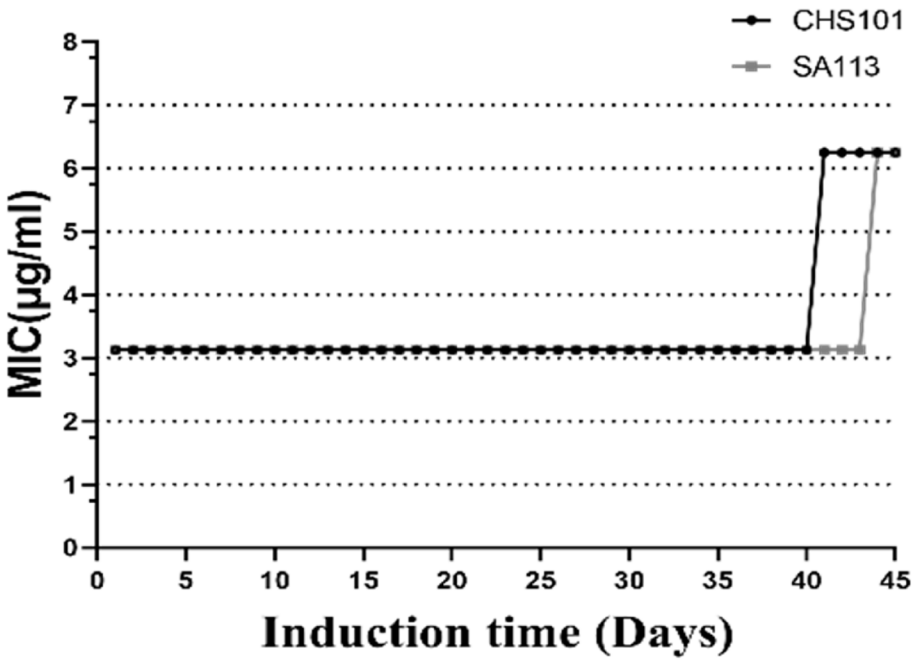

Fig. 5 The in vitro induction of *S. aureus* by Fingolimod exposure. The liquid culture of CH101 and SA113 strains were consecutively induced under Fingolimod pressure. After the 45-day induction, the individual clone was isolated and determined the MIC

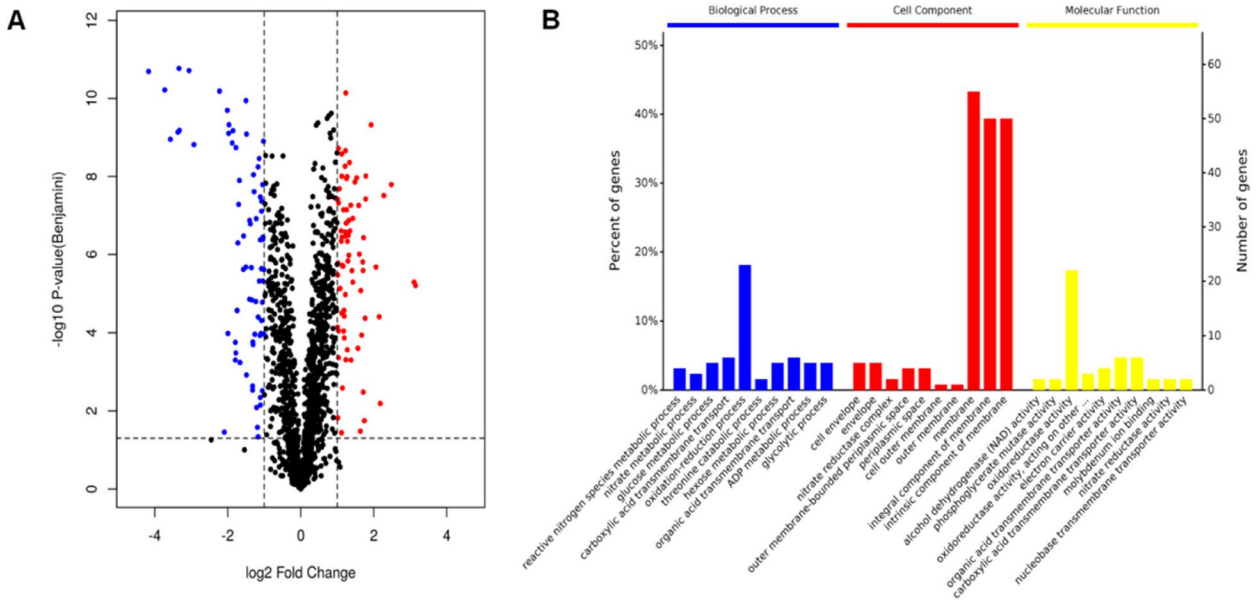

Fig. 6 Proteomic analysis of Fingolimod-treated *S. aureus* SA113. **A** Volcano plot. **B** GO clustering analysis

Fingolimod is well-tolerated with a favorable safety profile. In eukaryotic, the main mechanism of Fingolimod is involved in the inhibition of S1P signaling and selectively retained lymphocytes in the lymphoid organs by targeting sphingosine 1-phosphate (S1P) receptors [31, 32]. Additionally, several recent studies have also shown the antifungal, antiviral, and antibacterial effects of Fingolimod and its chemical homologues. Lu-Qi Wei et al. reported that Fingolimod exhibited a synergistic effect with Amphotericin B against diverse fungal pathogens due to the excessive accumulation of reactive oxygen species [33]. ZengZi Zhou et al. showed that Fingolimod

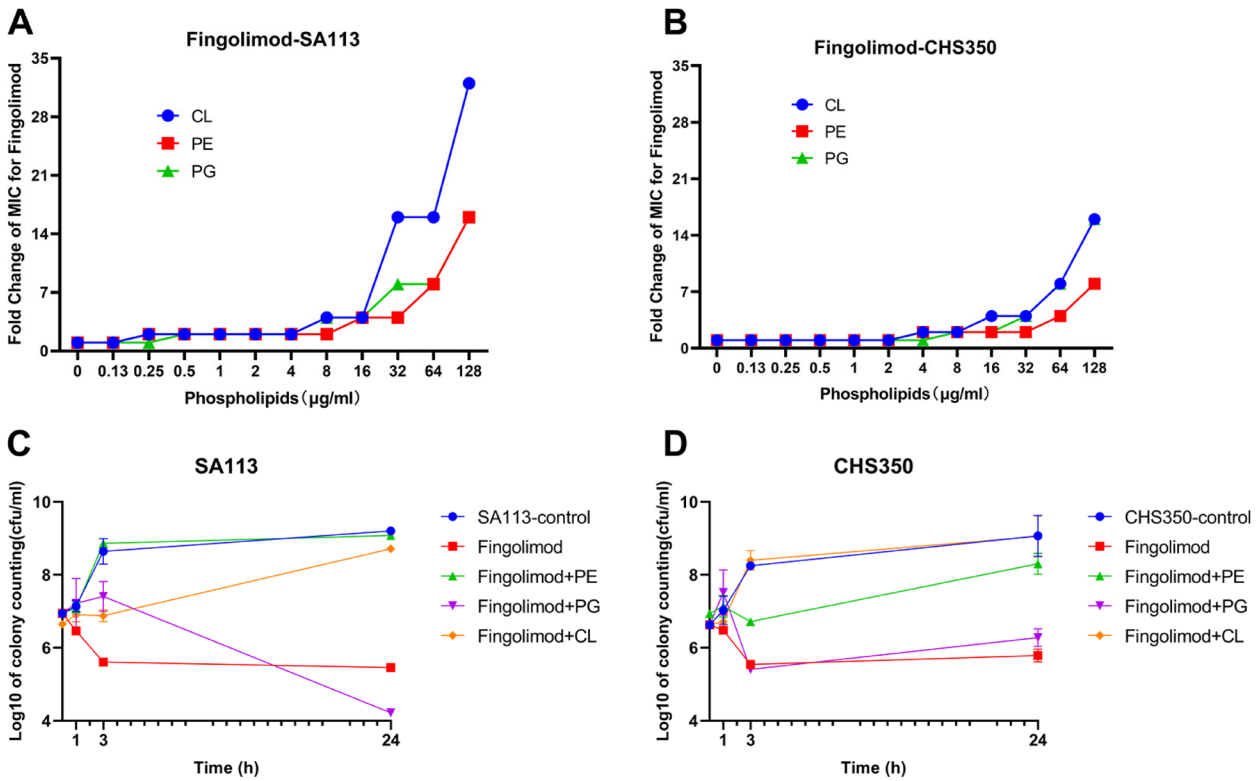

Fig. 7 Phospholipids inhibit antibacterial and bactericidal effects of Fingolimod. MICs and time-kill assay of Fingolimod against *S. aureus* SA113 (**A**) and (**C**), *S. aureus* CHS350 (**B**) and (**D**) were determined in the presence of exogenous phospholipids, including CL, PE, and PG. MIC Test: The concentrations of the three phospholipids were systematically varied from 0 to 128 μg/mL. This range was selected to comprehensively assess the dose-dependent influence of phospholipids on the MIC of Fingolimod against the two *S. aureus* strains. Time-kill assay: For the time-kill experiments, both SA113 and CHS350 cultures were adjusted to an initial density of 10^8 (CFU/mL. Subsequently, they were treated with Fingolimod at a concentration of 4× MIC, which was 12.5 μg/mL, and a fixed concentration of phospholipids at 64 μg/mL

inhibited Chlamydia dissemination from the upper genital tract to the gastrointestinal tract [34]. Recent two studies have demonstrated the robust inhibitory activity of Fingolimod and its homologues derivatives against a wide range of gram-positive bacteria and several gram-negative bacteria species. Moreover, the impact of these drugs on both planktonic growth and biofilm formation of *S. aureus* has been found. The chemical structure of Fingolimod was similar with to LuxR family quorum sensing (QS) and the mechanism of Fingolimod against Gram-negative bacteria has been speculated by inhibiting the QS [14, 15]. We found no alterations in the MIC of Fingolimod in the wild type and *luxS* deficiency of *Staphylococcus epidermidis* were examined, suggesting the impact of Fingolimod on *S. aureus* can't be explained by the hypothesis as different QS systems between Gram-positive and Gram-negative bacteria [35]. Our growth curve assays demonstrated that Fingolimod's bactericidal activity against *S. aureus* was significantly reduced at pH 8 compared to pH 6 (Fig. 5). We hypothesize that the alkaline environment facilitates the neutralization of the protonated NH^{3+} group by OH^- ions, thereby diminishing Fingolimod's efficacy. This observation aligns with the notion that the protonation of the $-NH_2$ group in fingolimod to $-NH^{3+}$ under acidic conditions is crucial for its bactericidal activity. For instance, sphingosine—a Fingolimod analog sharing the NH_2-bearing side chain—exhibits abolished bactericidal effects when its NH_2 group is chemically modified [36]. Our preliminary observations revealed strain-dependent MIC variations for Gram-negative pathogens: *E. coli*, *K. pneumoniae*, *and A. baumannii* exhibited MICs of 6.25 μg/mL in CAMHB and LB media, contrasting sharply with values >400 μg/mL in TSB and RPMI 1640. This phenomenon was absent in *P. aeruginosa* and all tested Gram-positive strains. The MICs of Fingolimod remained consistently stable across tested conditions, with Gram-positive pathogens *S. aureus* and *E. faecalis* maintaining an MIC of 6.25 μg/mL, while the Gram-negative bacterium *P. aeruginosa* exhibited sustained resistance with MIC values persistently exceeding 400 μg/mL (Supplementary Table 2). We hypothesize that media components (e.g.,

divalent cations, amino acids) may modulate Fingolimod's penetration through Gram-negative outer membranes, potentially via chelation or altered membrane stability. However, this warrants systematic investigation beyond the scope of our *Staphylococcus*-focused study.

Conclusions

Our data further supported the excellent antibacterial activity against planktonic cells and *persisters* in clinical strains of *S. aureus, E. faecalis,* and *S. agalactiae* with very low MIC values of 1.56–6.25 μg/mL. Fingolimod could significantly eliminate the *persisters*, inhibit biofilm formation and eradicate mature biofilm. In addition, disruption of the bacterial cell membrane permeability and envelope integrity was demonstrated by Fingolimod. In the presence of phospholipids, particularly cardiolipin, the antibacterial efficacy of the drug is significantly reduced. After Fingolimod exposure, proteomics suggests that the differential proteins are mainly related to redox functions and aspects of cell membrane composition and integrity. All in all, these findings point to the possibility that Fingolimod disrupts the cell membrane by acting on phospholipids. However, this study did not conclusively demonstrate direct interaction between Fingolimod and phospholipids. The precise molecular targets of Fingolimod remain to be elucidated in subsequent investigations. Additionally, the differential antibacterial efficacy of Fingolimod against Gram-negative bacteria under varying culture media conditions warrants further exploration.

Abbreviations

FDA	Food and Drug Administration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
TSB	Tryptic Soya Broth
MHB	Mueller–Hinton broth
DMSO	Dimethylsulfoxide
MIC	Minimum Inhibitory Concentration
CLSM	Confocal Laser Scanning Microscopy
PE	Phosphatidylethanolamine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
CL	Cardiolipin
QS	Quorum sensing

Supplementary Information

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Supplementary Material 1.

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Clinical trial number

Not applicable.

Authors' contributions

Yongpeng Shang: conceptualization (lead); formal analysis (equal); supervision (lead); writing original draft (equal). Yu Huang: formal analysis (equal) supervision (equal); writing— review and editing (equal). Fangyou Yu: Conceptualization (equal); formal analysis (equal) supervision (equal); writing— review and editing (equal). Qingyin Meng: methodology (equal). Zhijian Yu: practical methodology (equal); writing— review and editing (equal). Zewen Wen: methodology (equal); writing— review and editing (equal).

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Data availability

The whole-genome sequencing files of Fingolimod resistance CH101 and SA113 clone were deposited in the NCBI database with the biosample accession SAMN18385243/18385244 and SAMN18385245/183852436 and the reference sequence the parenteral isolate CH101 and SA113 with the biosample accession SAMN15745752/15745753 and SAMN15745758/15745759. Data will be made available on request.

Declarations

Ethics approval and consent to participate

Ethical review and approval were performed by the Medical Ethics Committee of Shanghai Pulmonary Hospital, School of Medicine (Ethical no. K23-088Y).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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