Efficacy of the novel oxazolidinone compound FYL-67 for preventing biofilm formation by Staphylococcus aureus

Sisi Wu†, Tao Yang‡, Youfu Luo‡, Xiaolu Li‡, Xian Zhang‡, Jianying Tang‡, Xiuying Ma‡ and Zhenling Wang*†

1Molecular Medicine Research Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China; 2State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China; 3Institute of Burn Research, Southwest Hospital, Third Military Medical University, Chongqing 400038, China

*Corresponding author. Tel: +86-28-85164063; Fax: +86-28-85164060; E-mail: wangzhenling@scu.edu.cn
†These authors contributed equally to this work.

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Objectives: Infections of hospitalized patients caused by biofilms formed by Staphylococcus aureus represent a major problem. Using in vitro and in vivo biofilm models, we evaluated the efficacy of the novel oxazolidinone FYL-67, by using linezolid (the only clinically approved oxazolidinone antibiotic) as a control, for inhibiting S. aureus biofilm formation.

Methods: Antibiofilm activity was determined using strains of methicillin-susceptible S. aureus and methicillin-resistant S. aureus. We studied the mechanism(s) and pharmacodynamics of antibiofilm activity as follows: (i) effects of pre- and post-exposure to FYL-67 or linezolid on biofilm formation; (ii) the effect of FYL-67 on biofilm structure; (iii) the role of FYL-67 in biofilm composition; (iv) effects on cell morphology; and (v) efficacy of FYL-67 and linezolid using an in vivo murine model of catheter infection.

Results: FYL-67 effectively inhibited biofilm formation using in vitro and in vivo assays.

Conclusions: Our data suggest that oxazolidinone compounds, such as FYL-67, may serve as antibiofilm agents.

Keywords: biofilm biomass, bacterial viability, antibiotics

Introduction

Infections caused by biofilms are a serious health problem with increasing morbidity, mortality and difficulties in effectively treating patients, particularly the elderly. According to the US NIH, biofilms account for >80% of human microbial infections. Biofilms form on indwelling medical implants such as catheters, artificial hips and contact lenses. Because of increasing resistance of biofilm-forming bacteria to antimicrobial agents, these infections can often only be treated by removal of the implant, which increases trauma to the patient and the cost of treatment.

Standard antimicrobial treatments usually are unsuccessful in eradicating biofilms, which leads to persistent infection. However, the first new oxazolidinone, linezolid, appears to be a promising treatment for biofilms formed by methicillin-resistant Staphylococcus aureus (MRSA) or catheter-associated infections. Moreover, linezolid exhibited strong antibacterial activity against formation of mature biofilms by methicillin-resistant Staphylococcus epidermidis biofilms compared with gentamicin and ciprofloxacin. Further, linezolid treatment completely eradicates bacterial biofilms of implants, thus preventing their surgical removal. Bacterial resistance to linezolid is difficult to induce, because it binds to ribosomes and inhibits protein synthesis. Bacteria have four to six copies of the gene encoding the 23S ribosomal RNA binding site, which means a lower frequency of resistance due to multiple mutations.

Although the exact mechanism by which linezolid inhibits biofilms is not completely understood, the above studies suggest that oxazolidinone compounds may effectively treat biofilms formed by MRSA.

FYL-67, a novel linezolid analogue with the morpholinyl ring replaced by a 4-(pyridin-2-yl)-1H-pyrazol-1-yl group, demonstrated increased activity against Gram-positive organisms in our previous study. Given its potency and broad spectrum of activity, it is important to determine how FYL-67 affects the ability of S. aureus to form and maintain a biofilm. To this end, we used models of biofilm formation by S. aureus to examine the activity of FYL-67, using linezolid as a control.

Materials and methods

Bacterial strains
Biofilm-producing strains of S. aureus (MSSA) ATCC 25923, MRSA ATCC 33591, MRSA ATCC 43300 and three
samples were sputter-coated with 200 Å of gold-palladium and viewed SEM by dehydration in a graded series of ethanol concentrations.

**Antibiotics**

Anhydrous (99.9% pure) FYL-67 was synthesized in our laboratory. Linezolid (≥99% pure, research grade) was purchased from Airsea Pharmaceutical Ltd, Taizhou, China.

**Media**

The media used included the following: tryptic soy broth (Qingdao Hope Bio-Technology, Qingdao, China) supplemented with 1% glucose and 2% NaCl for biofilm production; Mueller–Hinton broth (Qingdao Hope Bio-Technology) supplemented with calcium (25 mg/L) and magnesium (12.5 mg/L) for MIC and minimum biofilm eradication concentration (MBEC) determinations and the broth microdilution chequerboard technique; and tryptic soy agar (TSA) (Qingdao Hope Bio-Technology) for MBEC determinations and colony counts.

**Animals**

C57BL/6 male mice (6–8 weeks of age) were purchased from the Animal Centre of Sichuan University and experiments were performed according to protocols approved by the Ethics Review Committee for Animal Experimentation of Sichuan University. We used neutrophic mice for this study, a state that was induced by intraperitoneal injection of cyclophosphamide (Sigma–Aldrich, St Louis, MO, USA) for 5 days.

**MIC and MBC determinations**

The MICs of FYL-67 and linezolid were determined using the broth microdilution technique of the CLSI (formerly NCCLS). MBCs were determined at the end of the incubation period by removing two 10 μL samples from each well in which there was no visible growth and plating the samples onto TSA. The colonies were counted after overnight incubation at 37°C. The MBC was defined as the lowest concentration of antimicrobial that killed ≥99.9% of the initial inoculum.

**MBEC assays**

The MBEC was defined as the lowest concentration that prevented bacterial regrowth. MBECs for MSSA and MRSA biofilms were measured as previously described.

**Effect of FYL-67 on biofilm formation**

A modified microplate assay was used to evaluate the effects of FYL-67 and linezolid on biofilm formation. Briefly, 6 h ‘young’ biofilms and 24 h ‘mature’ S. aureus biofilms were induced in plates. After incubation, the medium was replaced and replaced with medium containing antibiotics at increasing concentrations (0.125× to 1× MIC). After incubation for another 24 h at 37°C, biomass formation was assessed visually by crystal violet staining and bacterial viability assays were conducted using resazurin staining as described previously.

**Analysis of biofilms using scanning electron microscopy (SEM)**

To facilitate microscopic observations, biofilms were cultivated overnight at 37°C on positively charged glass slides using biofilm growth medium inoculated with 10⁷ cfu/mL S. aureus and supplemented with FYL-67 (0.5× MIC), linezolid (0.5× MIC) or no drug. Biofilms were processed for SEM by dehydration in a graded series of ethanol concentrations. Samples were sputter-coated with 200 Å of gold-palladium and viewed in a Philips XL-20 SEM (FEI Company, Kassel, Germany) with an accelerating voltage of 20 kV. Digitized TIFF images were acquired.

**Confocal laser scanning microscopy (CLSM) analysis of extracellular polymeric substances (EPSs) present in biofilms**

Fluorescein isothiocyanate-labelled type IV concanavalin A (ConA; Sigma–Aldrich), which is specific for the α-mannopyranosyl and α-glucopyranosyl residues of EPSs, was used and propidium iodide (PI; Sigma–Aldrich) was used to label the nucleic acids of dead bacteria. Biofilm staining was performed as previously described. The samples were sequentially scanned using CLSM (Nikon Hydraulic Manipulator, Tokyo, Japan) with excitation at 488 and 543 nm to minimize channel cross-talk for colocalization analysis.

**Mouse model of biofilm contamination of catheters**

Biofilms were formed by S. aureus ATCC 25923 on 14 gauge Teflon intravenous catheters as described previously. Neutropenic mice were anaesthetized using ketamine (100 mg/kg) and the colonized catheters containing 10⁴ cfu/mL bacteria were implanted into 8–10 mm skin incisions on the flanks of each animal. The catheterized mice received daily caudal vein injections of FYL-67 or linezolid (10 mg/kg) for 3 days, starting 1 day post-colonization. Animals were sacrificed on day 4 and the imaging of catheters and viable cell count estimates was performed using SEM. Untreated and uncolonized implanted catheters were used as controls. Five animals were included in each group. Quadriceps femoris muscle, skin, heart, kidney and spleen were collected upon sacrifice and cfu counts were determined.

**Statistical analysis**

Statistical comparisons were performed using one-way analysis of variance (Excel 2000, Microsoft). A P value <0.05 denoted the presence of a statistically significant difference.

**Results**

**MIC, MBC and MBEC assays**

The in vitro activities of FYL-67 against MSSA and MRSA isolates are shown in Table 1. FYL-67 inhibited MRSA strains with MIC values ranging from 0.5 to 1 mg/L. The MBECs of FYL-67 and linezolid for MSSA and MRSA isolates ranged between 256 and 512 mg/L and 128 and 256 mg/L, respectively. The MICs of the antibiotics for the control strain S. aureus ATCC 25923 were within the range determined using CLSI.

**Impact of FYL-67 on biofilm formation**

Inhibition of young biofilms by FYL-67 was compared with inhibition by linezolid by analysing the absorbance of the crystal violet and resazurin stains for each biofilm (Figures 1 and 2). As showed in Figure 1, treatment with either FYL-67 or linezolid at all concentrations tested (0.125× to 1× MIC) reduced the biofilm mass of ATCC 25923 by 50% (P<0.01) compared with the control. In contrast, the biomass of MRSA strains was not decreased by FYL-67 or linezolid at 0.125× to 0.5× MICs. However, the addition of 1× MIC of FYL-67 reduced the biomass of the MRSA strains by 63%–72% (P<0.01). Linezolid treatment at the same concentration reduced the biomass by 59%–63% (P<0.05). For young biofilms formed by the three clinical strains, both FYL-67 and linezolid reduced biomass similarly, ranging from 20% to 35% at 0.5× MIC and from...
In vitro antimicrobial activity of FYL-67 and linezolid against MRSA and MSSA

<table>
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<th>Organism</th>
<th>Strain</th>
<th>Planktonic MIC (mg/L) FYL-67</th>
<th>Planktonic MBC (mg/L) FYL-67</th>
<th>MBEC (mg/L) FYL-67</th>
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<td></td>
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<td>2</td>
<td>512</td>
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<tr>
<td>MRSA</td>
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<td>4</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>ATCC 43300</td>
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<td>2</td>
<td>128</td>
</tr>
<tr>
<td>Clinical strains</td>
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<td>1</td>
<td>4</td>
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<td></td>
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50% to 80% (P<0.05) at 1x MIC. We simultaneously analysed the bactericidal activity of the different antibiotic compounds using the resazurin assay (Figure 2). Sub-MIC (0.125x, 0.25x and 0.5x MIC) amounts of the two antibiotics reduced bacterial viability within young MSSA biofilms by 50% (P<0.05). While both FYL-67 and linezolid were less potent against young MRSA biofilms at sub-MIC concentrations, viability was reduced by 50%–60% (P<0.05) at 1x MIC.

The maximum efficacies and potencies of the antibiotics for inhibiting mature biofilms were reduced. Moreover, FYL-67 exhibited a more potent ability to inhibit the biomass of all of the strains at 0.5x MIC compared with linezolid. FYL-67 was active against ATCC 25923 (52.1% inhibition, P<0.05), ATCC 33591 (43.3% inhibition, P<0.05), ATCC 43300 (42.7% inhibition, P<0.05), Au-1 (32.9% inhibition, P<0.05), Au-2 (34.8% inhibition, P<0.05) and Au-3 (41.3% inhibition, P<0.05). However, linezolid only reduced the biomass of ATCC 25923 by 25%, a much more modest effect than FYL-67 (Figure 1). FYL-67 decreased the number of viable bacteria in MSSA biofilms by 3%, 19% and 65% (P<0.05) at 0.25x, 0.5x and 1x MIC, respectively, compared with 0.5%, 5.4% and 17.1%, respectively, for the same concentrations of linezolid. For ATCC 33591, ATCC 43300 and the clinical strains, linezolid was equally effective as FYL-67 in suppressing bacterial viability at 0.5x MIC, with inhibitory effects ranging from 30% to 45% (Figure 2). These results indicate that FYL-67 inhibited both young and mature biofilms more potently than linezolid. Moreover, our results show that antibiotic activity depends on biofilm maturity and the bacterial strain. We determined the ability of sub-MIC amounts of FYL-67 and linezolid to prevent the formation of 6 and 24 h biofilms by adding the antibiotics at the beginning of the incubation period. A potent antibiofilm effect was observed for FYL-67 compared with linezolid, with biofilm biomass and viability effectively inhibited by FYL-67 (Figures S1 and S2, both available as Supplementary data at JAC Online). We also investigated the effects of FYL-67 and linezolid at sub-MIC concentrations (0.5x MIC) on the attachment of S. aureus, but found no significant difference among the two drugs and the control (Figure S3b).

CLSM and SEM analyses of the efficacy of FYL-67 against S. aureus biofilm

Because FYL-67 inhibited biofilm formation, we were interested in the ability of FYL-67 to prevent bacterial aggregation and EPS production. The efficacy of FYL-67 for eliminating bacterial aggregation and EPS production were analysed using CLSM and SEM. We characterized biofilms formed on glass coverslips treated with FYL-67 or linezolid at 0.5x MIC, which were effective concentrations for eradicating S. aureus biomass (Figure 3 and Figure S4) (available as Supplementary data at JAC Online). SEM images of standard and clinical isolates of MRSA revealed a relatively uniform thickness throughout the biofilm matrix in the well biofilms consisting of multilayered cell clusters after 24 and 48 h incubation at 37°C. In contrast, young ATCC 25923 biofilms were moderately adherent and its mature biofilms comprised aggregates of cells without forming a more multilayered structure. Smaller clusters of bacteria formed after treatment with FYL-67 compared with linezolid. Treatment with either FYL-67 or linezolid resulted in similar morphological changes in young and mature biofilms and the cells appeared discoid and biconcave with bilateral central depressions.

We used CLSM to assess the effect of FYL-67 at 0.5x MIC on the components of the biofilms. All untreated young biofilms formed by S. aureus remained relatively small and patchy with many dead cells at the centre of the microcolonies, particularly those biofilms formed by MRSA and clinical strains (Figure 3). All untreated strains formed intact mature biofilms where the residual cells proliferated to occupy the vacuolar architecture, renewing the biofilm where any dead cells were present (Figure S4). Young and mature biofilms formed by strains ATCC 25923, ATCC 33591 and ATCC 43300 treated with FYL-67 harboured a small fraction of dead bacteria and the amount of EPSs was reduced substantially compared with the untreated group. In particular, more EPSs and dead bacteria were removed by FYL-67 treatment compared with linezolid. FYL-67 and linezolid had similar effects on the biofilms produced by the three clinical isolates.

Therapeutic efficacy of FYL-67 for treating catheter-associated infection in a murine model

A mouse model of catheter infection was used to evaluate the in vivo antibiofilm activity of FYL-67. Bacteria were cultivated in vitro on implantable catheters and induced to form biofilms and the resazurin assay (Figure 2).
in mice. Before implantation, SEM analysis was performed to verify the formation of biofilms on the catheters (Figure 4a). The effects of FYL-67 or linezolid on the catheter biofilms are shown in Figure 4(c and d). FYL-67 treatment removed surface bacteria more effectively than linezolid. Further, bacterial loading of catheters and tissues from mice treated with FYL-67 was calculated and compared with results from animals treated with linezolid (Figure 4e). The numbers of viable bacteria remaining in the

Figure 1. Effects of FYL-67 or linezolid on young and mature biofilm biomass quantified by crystal violet (CV) assay. Biofilms were grown in 96-well plates for 6 h (young biofilm) or 24 h (mature biofilm) and then biofilm culture medium was removed and replaced by the same medium (control) or medium containing antibiotics at increasing concentrations (0.125× to 1× MIC). Biofilms were then reincubated for 24 h at 37°C. Each bar represents 10–12 assays. *P<0.05 and **P<0.01, compared with the control biofilms. #P<0.05, comparing FYL-67-treated with linezolid-treated biofilms at the same concentration. LZD, linezolid.
Catheters were lower by factors ranging from $10^3$ to $10^4$ in mice treated with FYL-67 compared with those treated with linezolid. The number of cfu on the skin decreased significantly ($P < 0.01$) after FYL-67 treatment from $10^{10} - 10^7$ cfu and $10^{10} - 10^{8.5}$ cfu for linezolid-treated mice and reduced the number of viable bacteria in the kidney, quadriceps femoris muscle and spleen by a factor of $\approx 100$ compared with linezolid treatment. The number of cfu ($10^5$) recovered from heart tissue did not significantly differ between the two treatments, but was lower by a factor of 10 compared with the control (solvent treated).

Figure 2. Bacterial viability in young and mature biofilm treated with FYL-67 or linezolid was quantified using the redox indicator resazurin. The biofilms were exposed to various concentrations ($0.125 \times$, $0.25 \times$, $0.5 \times$ and $1 \times$ MIC) of FYL-67 or linezolid. Biofilms were grown in 96-well plates and quantified by resazurin staining. Each bar represents six assays. *$P < 0.05$ and **$P < 0.01$, compared with the control biofilms. # $P < 0.05$, comparing FYL-67-treated with linezolid-treated biofilms at the same concentration. LZD, linezolid.
Discussion

Two distinct characteristics of biofilms cause problems associated with human biofilm infections. First, biofilms are highly resistant to treatment with antimicrobial agents and to immune killing and clearance. In the present study, S. aureus biofilms were resistant to concentrations of FYL-67 or linezolid that were 100–200× higher than those that inhibited the growth of planktonic cells (Table 1). Second, biofilms shed bacteria or biofilm aggregates into surrounding tissues and the circulatory system. These cells may cause acute illness, which recurs despite vigorous treatment with antimicrobials. These properties are the focus of research into the mechanisms of biofilm formation, particularly the mechanisms that are responsible for bacterial attachment and proliferation.

In the present study, we show that the new oxazolidinone antibiotic FYL-67 inhibited mature biofilm formation by three ATCC strains and three clinical isolates in vitro and in vivo. We used a microtitre plate biofilm model to assess the ability of FYL-67 to inhibit biofilm formation. Evidence indicates that most antistaphylococcal agents are ineffective, because they do not inhibit the formation of mature biofilms. For example, findings that sub-MIC concentrations of vancomycin stimulate the growth of biofilms reveal the deficiencies of vancomycin as a first-line anti-MRSA agent and account for the increasing number of resistant strains. Here, we show that FYL-67 effectively kills MSSA and MRSA isolates in a biofilm. Further, low concentrations of FYL-67 were clearly more effective than linezolid at equipotent concentrations, suggesting that factors other than higher intrinsic activity contribute to efficacy.

We show here that the inhibitory activity of the antibiotics decreases with maturation of the biofilm (Figures 1 and 2). S. aureus biofilms were more effectively inhibited by FYL-67 than by linezolid following prophylactic treatment (Figure S1 and Figure S2). In addition to the ability to form a mature biofilm, the specific bacterial strain also clearly influences antibiotic activity. Thus, the MSSA strain ATCC 25923 formed moderately adherent biofilms and MRSA strains (ATCC 33591 and ATCC 43300) and three clinical isolates formed fully established biofilms (Figure S3a). The SEM images support the results obtained using

![Figure 3. SEM and CLSM analysis of young S. aureus strains biofilms. SEM of young S. aureus biofilms formed by S. aureus cultivated on glass cover slips for 6 h, then young biofilms exposed to 0.5× MIC FYL-67 or 0.5× MIC linezolid for 24 h. Scale bar = 2 μm. CLSM analysis of young S. aureus biofilms exposed to FYL-67 or linezolid at 0.5× MIC. CLSM of young biofilms formed by MRSA and MSSA. S. aureus was cultivated on 6-well plates for 6 h, then young biofilms exposed to either FYL-67 or linezolid for 24 h. EPSs were detected by fluorescence in the green spectrum as a result of the binding of ConA-green. The red fluorescence indicates the dead cells stained by PI, a dye that stains nucleic acid. Scale bar = 10 μm. LZD, linezolid.](image-url)
crude crystal violet staining of biofilm biomass and highlight the effect of FYL-67 against biofilm formation by *S. aureus* (Figure 3 and Figure S4).

At subinhibitory concentrations, both linezolid and FYL-67 altered the composition of *S. aureus* biofilms. Biochemical analyses revealed a significant reduction of carbohydrate (polysaccharides) and protein content but not a significant reduction in extracellular DNA (Figure S5, available as Supplementary data at JAC Online). Polysaccharides account for a major fraction of the EPS matrix.31 As well as CLSM to visualize the complex networks present within the *S. aureus* biofilms, biochemical analyses were performed for independent verification (Figure S6, available as Supplementary data at JAC Online). Exopolysaccharides in biofilms contribute to cellular recognition and intracellular adhesion.32 For example, clinical strains often produce polysaccharide intracellular adhesion (PIA) molecules, which play a crucial role in biofilm generation, and they express high levels of the *ica* genes involved in PIA synthesis.33 However, because of limitations in our ability to precisely label exopolysaccharides in biofilms, it is very difficult to identify specific changes in each kind of exopolysaccharide. Sub-MIC amounts of either FYL-67 or linezolid altered exopolysaccharide levels formed by *S. aureus*, causing a change in biofilm structure and impairing attachment.

Proteins can account for considerable amounts of the mass of the biofilm matrix that far exceeds the polysaccharide content. Extracellular proteins contribute to biofilm formation by staphylococci, including the accumulation-associated protein of *S. epidermidis* and the biofilm-associated protein of *S. aureus*. These large proteins are expressed on the bacterial cell surface and promote biofilm formation by initiating colonization of cells on tissues or organs.34
implants. We show here that treatment with FYL-67 or linezolid resulted in a significant reduction of protein in the EPSs formed by MRSA compared with MSSA strains. In a recent study of clinical S. aureus isolates, biofilm production by MSSA strains differed from that of MRSA strains, which was dependent on the murein hydrolase Atl (FnBP) in the latter. Because of the potent inhibitory effects of FYL-67 and linezolid on protein production by S. aureus in biofilms, we speculate that these antibiotics are general protein synthesis inhibitors. This finding agrees with a study demonstrating that subinhibitory concentrations of linezolid impair staphylococcal adherence in vitro.

In the current study, the host defence system was eliminated in experimental mice by treatment with cyclophosphamide before implantation of catheters. This allowed direct determination of the therapeutic efficacy of the drugs without the confounding influence of the host immune response. FYL-67 and linezolid inhibited biofilm-associated infection and reduced the bacterial load in organs including quadriceps femoris muscle, skin, heart, kidney and spleen that were far removed from the location of the primary catheter. This suggested powerful inhibition of detachment, adherence or both of bacteria to the host cell surface.

At equipotent concentrations, bacterial loading in organs from mice treated with FYL-67 was lower compared with animals treated with linezolid, except in the heart (Figure 4). We speculate that this was partly because of differences between the two oxazolidinone antibiotics, particularly regarding their tissue distribution and protein binding. These differences require further investigation. Our data indicate that administration of oxazolidinone antibiotics prevents bacteremia or infection of remote sites and that FYL-67 is more efficacious than linezolid for these purposes. Our observations suggest that treating catheter infections with FYL-67 may completely eradicate the entire biofilm as well as non-contiguous fragments and therefore avoids surgical removal of an implant.

SEM observations further clarified the morphology of cells both embedded in biofilms and in the planktonic state (Figure S7, available as Supplementary data at JAC Online). Linezolid and FYL-67 caused dimple-like dents to form on the cell surface, regardless of whether the cells were part of a biofilm or in the planktonic state. These observations are consistent with those reported for vancomycin, the standard antibiotic for treatment of hospital-acquired infections. FYL-67 has a structure similar to that of linezolid. We predict that compound FYL-67 has the same ribosomal target as linezolid and we plan to confirm this in future research.

Our findings indicate that FYL-67 shows promise as an effective antibiotic for treating infections caused by the formation of biofilms by MRSA. Moreover, we show here that oxazolidinone drugs, particularly FYL-67, prevent the maturation of biofilms formed by S. aureus by inhibiting the production of the carbohydrate and protein components of the EPSs. Future studies will employ FYL-67 as a scaffold for developing more efficacious oxazolidinones and will investigate whether FYL-67 can synergize with other antibiotics to prevent the formation and maturation of biofilms.

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Transparency declarations
None to declare.

Supplementary data
Figures S1 to S7 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References
FYL-67 inhibits S. aureus biofilms


