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ORIGINAL ARTICLE

# Antibacterial and anti-biofilm activities of thiazolidione derivatives against clinical staphylococcus strains

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**Both *Staphylococcus aureus* and *Staphylococcus epidermidis* can form biofilms on natural surfaces or abiotic surfaces, such as medical implants, resulting in biofilm-associated diseases that are refractory to antibiotic treatment. We previously reported a promising antibacterial compound (Compound 2) and its derivatives with bactericidal and anti-biofilm activities against both *S. epidermidis* and *S. aureus*. We have further evaluated the antibacterial activities of four Compound 2 derivatives (H2-38, H2-39, H2-74 and H2-81) against 163 clinical strains of *S. epidermidis* and *S. aureus*, including methicillin-susceptible and methicillin-resistant strains, as well as biofilm-forming and non-biofilm-forming strains. The four derivatives inhibited the planktonic growth of all of the clinical staphylococcal isolates, including methicillin-resistant *S. aureus* and methicillin-resistant S. *epidermidis* and displayed bactericidal activities against both immature (6 h) and mature (24 h) biofilms formed by the strong biofilm-forming strains. The derivatives, which all target YycG, will help us to develop new antimicrobial agents against multidrug-resistant staphylococci infections and biofilm-associated diseases.**
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**Keywords:** antibacterial; anti-biofilm activity; methicillin-resistant *Staphylococcus aureus*; minimal inhibitory concentration; $MIC_{50}$; *Staphylococcus epidermidis*

## INTRODUCTION

Staphylococci are major causes of hospital-acquired infections that are associated with high morbidity. Most stains of *Staphylococcus aureus* and *Staphylococcus epidermidis* that cause nosocomial infections are antibiotic-resistant.[1,2] Methicillin-resistant *S. aureus* (MRSA) has emerged as a cause of potentially lethal infections, and it can increase morbidity, length of hospital stay and hospital costs.[3,4] Moreover, the number of multidrug-resistant MRSA and methicillin-resistant *S. epidermidis* (MRSE) strains that exhibit resistance to antibiotics, such as aminoglycosides, macrolides and lincosamides, has been on the rise.[5] In addition, the ability of *S. epidermidis* and *S. aureus* to form biofilms on natural and abiotic surfaces, such as medical implants, has made these pathogens a major cause of refractory biofilm-associated infections because the biofilm bacteria show phenotypic resistance to antibiotics.[6,7]

The bacteria embedded in a biofilm exist in a low metabolic state or at a stationary growth phase, and they are much less susceptible to the host immune system and to antimicrobial agents. Bacteria in a biofilm are 10–1000 times more resistant to the effects of antimicrobial agents.[8,9] Bacterial biofilms account for more than 80% of all microbial infections in humans,[10] and 60% of nosocomial infections are related to the formation of biofilms on implantable medical devices.[11] The colonization of staphylococci on implanted medical devices and formation of biofilm can result in chronic infections that are difficult to eradicate, thereby causing increased trauma to the patient and increased cost of treatment.[12,13]

Conventional antimicrobial agents have limited effectiveness against biofilm-related infections,[12] which increases the emergence of multidrug-resistant staphylococci. This and the increased use of implanted medical devices, such as vascular catheters and joint prostheses, together drive the need for developing new types of antibiotics to effectively combat multidrug-resistant and biofilm-associated diseases. Two-component systems (TCSs), which exist in most bacteria, play important roles in sensing and responding appropriately to a wide range of environmental signals, and they have been considered to be potential targets for antimicrobial therapy.[14] The YycGF TCS, which was discovered in low G+C gram-positive bacteria, including *S. aureus* and *S. epidermidis*, is an essential regulatory system for cell wall metabolism and is highly conserved,[15] thus making it an attractive drug target. YycG is a sensor histidine-kinase, and YycF is the cognate response regulator. Inhibitors targeting the YycG kinase or the YycF regulator have been reported for *B. subtilis* and *S. aureus*.[16–19]

We previously reported a compound (Compound 2) that targets the HK domain of the *S. epidermidis* YycG and exhibits bactericidal and anti-biofilm activities.[20] The structure of Compound 2 was optimized by substituting different functional groups, and a series of derivatives were designed and synthesized. Several derivatives with more

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effective antibacterial activity were then screened out.[21–23] In this study, 163 clinical strains of *S. aureus* and *S. epidermidis* were collected from three tertiary hospitals in eastern China and used to evaluate the anti-bacteria activities of the newly synthesized derivatives of Compound 2 (H2-38, 3-{5-{{3-(4-chlorophenyl)-2-[(4-chlorophenyl) imino]-4-oxothiazolidin-5-ylidene}methyl}furan-2-yl}benzoic acid; H2-39, 4-{5-{{3-(4-chlorophenyl)-2-[(4-chlorophenyl)imino]-4-oxothiazolidin-5-ylidene}methyl}furan-2-yl}benzoic acid; H2-74, 2-{4-{{3-(4-chlorophenyl)-2-[(4-chlorophenyl)imino]-4-oxothiazolidin-5-ylidene}methyl}phenoxy}acetic acid; and H2-81, 4-{5-{{3-(4-fluorophenyl)-2-[(4-phenyl)imino]-4-oxothiazolidin-5-ylidene}methyl} thiophene-2-yl}benzoic acid) against the clinical staphylococcal isolates under planktonic and biofilm growth conditions.

## MATERIALS AND METHODS

### Clinical bacterial strains and culture media

A total of 163 staphylococci, each from an individual patient, were retrospectively collected from three tertiary teaching hospitals in three provinces in eastern China (ZheJiang, Jiangsu and Shanghai) from July 2011 to January 2012. The isolates were identified by the VITEK 2 compact automated system (bioMerieux SA, Lyon, France) and stored in trypticase soy broth (Oxoid, Sydney, NSW, Australia) with 15% glycerol at −80 ℃ until they were used for the present study.[24]

*S. aureus* (ATCC2 5923), *S. aureus* (ATCC 49230) and *S. epidermis* (ATCC 35984) strains were tested by the broth microdilution minimum inhibitory concentration (MIC) test. The biofilm-forming strain, *S. epidermis* ATCC 35984, and the biofilm-negative strain, *S. epidermis* ATCC 12228, were used in the biofilm formation assay as controls. All staphylococci were cultured in tryptic soy broth medium (TSB; Oxoid Ltd, Basingstoke, UK). Mueller-Hinton broth (Oxoid Ltd) was used for the MIC assay.

### Biofilm formation assay of the clinical staphylococcal isolates

Biofilm formation of the clinical *S. aureus* and *S. epidermis* isolates was detected by a semi-quantitative assay in 96-well polystyrene microtiter plates. Overnight cultures of clinical strains were diluted 1:200 in TSB (Oxoid Ltd) containing 1% glucose and dispensed into 96-well plates (200 μL/well). After static incubation at 37 ℃ for 24 h, the plates were washed gently three times with phosphate-buffered saline to remove unattached bacteria, dried for 10 min at 60 ℃, fixed with methanol and stained with 2% (w/v) crystal violet for 15 min at room temperature. Crystal violet staining was scanned at 570 nm using a 96-well plate spectrophotometer (DTX 880 Multimode Detector, Beckman Coulter, Brea, USA) to determine the optical density of the stained biofilms. The *S. epidermidis* (ATCC 35984) and *S. epidermidis* (ATCC 12228) strains were used as positive and negative biofilm-forming controls in the experiments, respectively.[25] The clinical strains were divided into four groups according to their biofilm-forming ability as measured by OD values at 570 nm ($OD_{570}$). The cutoff OD value (ODc) was defined as three standard deviations above the mean OD of the negative control. The OD value of a tested strain was expressed as follows: OD=average $OD_{570}$−ODc. The strains were divided into the following groups: $OD_{570} \leqslant$ ODc=no biofilm producer (−); ODc$<OD_{570} \leqslant$ 2×ODc=weak biofilm producer (+); 2×ODc$<OD_{570} \leqslant$4×ODc= moderate biofilm producer (++); and 4×ODc$<OD_{570}$=strong biofilm producer (+++).[26]

### MIC assay of the derivatives against clinical staphylococcal isolates

The derivatives of Compound 2 used in the experiment (H2-38, H2-39, H2-74 and H2-81) were designed by substituting functional groups while keeping the core thiazolidione structure intact. The compounds were synthesized by Professor Han's group at Nanjing University of Technology.

Broth micro-dilution testing was used to determine the MICs in accordance with the guidelines provided by the American Clinical and Laboratory Standards Institute.[27] A range of different concentrations (0.8–200 μM) of derivative in Mueller-Hinton broth (Oxoid Ltd) was prepared and dispensed into a microtiter plate (100 μL per well), and 100 μL of bacteria ($10^6$ colony-forming units (CFU)/mL) was then added into each well resulting in final concentrations of the derivative from 0.4–100 μM. After a 12-h incubation at 37 ℃, the MIC value was determined as the lowest concentration of the derivative to completely inhibit bacterial growth as observed by naked eye.[28] The *S. aureus* (ATCC 25923), *S. aureus* (ATCC 49230), *S. epidermis* (ATCC 35984) and *S. epidermis* (ATCC 12228) strains were used as quality control strains. All tests were performed in triplicate and repeated three times.

### Effects of the derivatives on 6-h biofilms of clinical strains

The inhibitory effects of H2-38 and H2-81 on 6-h biofilms of the clinical staphylococcal isolates[19] were evaluated by a semiquantitative plate assay.[26] All of the tested strains were strong biofilm-forming strains, including eight MRSE, four methicillin-susceptible *S. epidermidis* (MSSE), five MRSA and two methicillin-susceptible *S. aureus* (MSSA) strains. The bacteria were statically incubated in polystyrene 96-well plates with TSB medium containing 0.25% glucose at 37 ℃ for 6 h. After the removal of planktonic cells, 12.5 μM H2-38 and H2-81 in fresh TSB were separately added to each well and incubated for another 16 h at 37 ℃. The wells were then washed gently three times with phosphate-buffered saline, fixed with methanol, and stained with 2% (w/v) crystal violet. The $OD_{570}$ of the wells was determined using a 96-well plate spectrophotometer (DTX880; Beckman Coulter, USA).

### Visualization of 24-h biofilms by confocal laser scanning microscopy

Overnight cultures of the selected clinical strains diluted at a ratio of 1:200 were inoculated in cell culture dishes (WPI, Florida USA) and incubated at 37 ℃ for 24 h. After removal of non-adherent cells with phosphate-buffered saline, the biofilms were stained using the Live/Dead BacLight Viability Kit (BacLight; Molecular Probes, Eugene, Oregon, USA), which employs SYTO9 and propidium iodide (SYTO9/PI) and they were subsequently analyzed with a Leica confocal laser scanning microscope (TCS SP5; Leica, Heidelberg, Germany). A series of images was acquired at 1 μm intervals in the Z section to measure the biofilm thickness in microns. IMARIS 7.0.0 software (Bitplane, Zurich, Switzerland) was used to create a three-dimensional view of the formed biofilms. A minimum of six representative optical fields were randomly selected and observed per specimen.[29]

To evaluate antibacterial effects of the H2-38 and H2-81 derivatives on 24-h biofilms, six strains were randomly selected out of the 19 strong biofilm-forming strains (MRSA 1000234, MRSA 916054, MSSA 1815, MRSE 1020081, MRSE 915296 and MSSE 914111). Overnight cultures of the bacteria (diluted 1:200) were inoculated in cell culture dishes (23 mm diameter) with glass bottoms (WPI) and incubated at 37 ℃ for 24 h. After removal of the suspension cultures, four-fold MIC concentrations of the derivatives in fresh TSB were added and incubated at 37 ℃ for an additional 12 h. The biofilms were then stained with the Live/Dead BacLight Viability Kit and visualized by a Leica TCS SP5 confocal laser scanning microscope. Viable cells in the biofilms exhibited green fluorescence, and dead cells exhibited red fluorescence. IMARIS 7.0.0 software (Bitplane, Zurich, Switzerland) was used to create the three-dimensional structural

**Table 1 The biofilm-forming abilities of the clinical staphylococcal isolates**

| | Biofilm-negative[b] (number (%)) | Biofilm-positive[b] (number (%)) | | |
|---|---|---|---|---|
| Strains[a] | - | + | ++ | +++ |
| MRSA | 32 (57.6) | 15 (27.3) | 3 (6.0) | 5 (9.1) |
| MSSA | 22 (54.1) | 13 (33.3) | 3 (8.3) | 2 (4.3) |
| MRSE | 16 (37.2) | 11 (25.7) | 9 (20.0) | 8 (17.1) |
| MSSE | 10 (41.6) | 7 (29.2) | 3 (12.5) | 4 (16.7) |

[a] Biofilm formation by the clinical isolates was assessed by semiquantitative assay with microtiter plates.

[b] The clinical strains were classified into four groups according to their abilities to form biofilms as measured by $OD_{570}$. The cutoff OD value (ODc) was 0.16. The four groups were as follows: no biofilm producer (−), $OD_{570} \leqslant 0.16$; weak biofilm producer (+), $0.16 < OD_{570} \leqslant 0.32$; moderate biofilm producer (++), $0.32 < OD_{570} \leqslant 0.64$; and strong biofilm producer (+++), $OD_{570} > 0.64$.

images of biofilms, and the percentages of live bacteria in the total bacterial counts were calculated by ImageJ (Wayne Rasband, NIH, Bethesda, MD, USA).

### Statistical analysis

The average biofilm thickness was calculated from the six views for each clinical isolate and reported as the mean with standard deviations. Fisher's exact test was conducted for the anti-staphylococcal activities of the derivatives with respect to the different strains.

## RESULTS

### Characteristics of the clinical staphylococcal isolates

The 163 clinical isolates comprising *S. aureus* (95) and *S. epidermidis* (68) were primarily isolated from blood, sputum and wound discharges (Supplementary Table S1). Of the isolates, 99 were methicillin-resistant accounting for 60.7% of the clinical staphylococcal isolates. The 95 *S. aureus* isolates included 40 MSSA and 55 MRSA strains. The 68 *S. epidermidis* isolates consisted of 44 MRSE and 24 MSSE strains. All methicillin-resistant staphylococcal strains were not susceptible to penicillin. The MRSA and MRSE strains showed much higher rates of multidrug resistance compared with the MSSA and MSSE strains. The 55 MRSA strains were characterized as follows: 36 (65.4%) were not susceptible to tetracycline and levofloxacin; 47 (85.4%) were resistant to erythromycin; 37 (67.3%) were resistant to gentamycin; 18 (32.7%) were resistant to rifampicin; and 19 (34.5%) were resistant to sulfadiazine and trimethoprim (SMZ-TMP) (Supplementary Table S2).

The microtiter plate assay of biofilm formation by the clinical isolates was done using the non-biofilm-forming *S. epidermidis* ATCC 12228 strain as a negative control, which gave an $OD_{570}$ cutoff value (ODc) defined as 0.16. The clinical strains were classified into four groups as follows: non-biofilm producer ($OD_{570}$ less than or equal to 0.16); weak biofilm producer ($0.32 \geqslant OD_{570} > 0.16$); moderate biofilm producer ($0.64 \geqslant OD_{570} > 0.32$); and strong biofilm producer ($OD_{570} > 0.64$). Of the 95 *S. aureus* isolates, 41 strains produced biofilms, and 7 isolates were strong biofilm producers. Of the 68 *S. epidermidis* isolates, 42 isolates produced biofilms, and 12 isolates were strong biofilm producers (Table 1). There was no statistically significant difference between the biofilm-forming abilities of the methicillin-resistant and methicillin-susceptible staphylococcal isolates ($P > 0.05$).

Confocal microscopy measurements of biofilm thickness showed the following results: the strong biofilm producers formed dense biofilms with thicknesses ranging from 9 to 14 μm; the moderate biofilm producers produced biofilms that were 5–8 μm thick; and the weak biofilm producers produced biofilms that were 2–4 μm thick. With those strains that did not produce biofilms, only scattered microcolonies at the bottom of the dishes were observed (Figure 1).

### Antimicrobial efficacy of H2 derivatives against clinical MRSA and MRSE isolates

Four of the newly synthesized derivatives of Compound 2 (H2-38, H2-39, H2-74 and H2-81) had strong antimicrobial efficacy against staphylococcus (*S. epidermis* ATCC 35984, *S. aureus* ATCC 25923 and *S. aureus* ATCC 49230),[23] and their anti-bacterial activities against 163 clinical staphylococcal isolates (99 of which were methicillin-resistant) were evaluated in this experiment. Most MIC values of the four derivatives were in the range of 0.75–25 μM. Both the methicillin-resistant and methicillin-susceptible strains of *S. aureus* and *S. epidermidis* were susceptible to the four derivatives (Table 2).

H2-38 and H2-81 inhibited the growth of all the 163 staphylococcal isolates (68 *S. epidermidis* strains and 95 *S. aureus* strains) at concentrations $\leqslant$12.5 μM. H2-39 and H2-74 inhibited the growth of 158 isolates (68 *S. epidermidis* strains and 95 *S. aureus* strains) at concen-

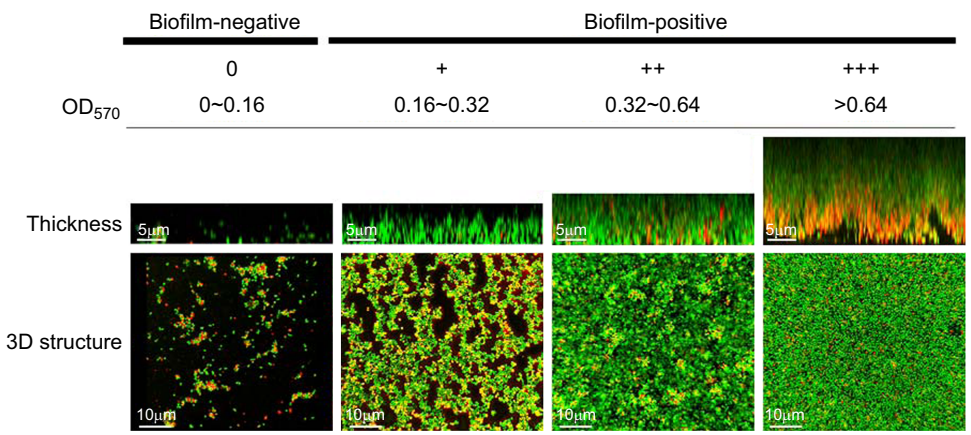


**Figure 1** Representative biofilms of the clinical staphylococcal strains. The clinical staphylococcal strains were classified into four groups according to their biofilm-forming ability. After a 24-h incubation, the biofilms were observed by confocal microscopy with Live/Dead staining. A series of images were acquired at 1 μm intervals along the Z section, and three-dimensional biofilm architectures were constructed by IMARIS 7.0.0.

**Table 2 MIC values of the four derivatives against the clinical staphylococcal isolates**

| Strains | Derivatives[a] | The range of MIC[b] (μM) | $MIC_{50}$[c] (μM) | $MIC_{90}$[c] (μM) |
|---|---|---|---|---|
| MRSE (44) | H2-38 | 0.75–12.5 | 3.13 | 6.25 |
| | H2-39 | 3.125–12.5 | 3.13 | 6.25 |
| | H2-74 | 3.125–12.5 | 6.25 | 12.5 |
| | H2-81 | 0.75–6.25 | 3.13 | 6.25 |
| MSSE (24) | H2-38 | 0.75–6.25 | 3.13 | 6.25 |
| | H2-39 | 1.5–12.5 | 3.13 | 6.25 |
| | H2-74 | 3.13–25 | 6.25 | 12.5 |
| | H2-81 | 0.75–12.5 | 3.13 | 6.25 |
| MRSA (55) | H2-38 | 0.75–12.5 | 6.25 | 12.5 |
| | H2-39 | 1.5–25 | 6.25 | 12.5 |
| | H2-74 | 1.5–25 | 6.25 | 12.5 |
| | H2-81 | 0.75–12.5 | 3.13 | 6.25 |
| MSSA (40) | H2-38 | 0.75–12.5 | 3.13 | 6.25 |
| | H2-39 | 1.5–12.5 | 3.13 | 6.25 |
| | H2-74 | 3.125–100 | 6.25 | 12.5 |
| | H2-81 | 0.75–12.5 | 3.13 | 6.25 |

[a] Stock solutions of the compounds were prepared in 0.1% DMSO.
[b] MIC represents the minimal inhibitory concentration of the derivatives against the test strains as determined by broth micro-dilution testing on a microtiter plate according to the standards of the American Clinical and Laboratory Standards Institute.
[c] The $MIC_{50}$ is the concentration below which 50% of the clinical isolate MIC values lie, and $MIC_{90}$ is the concentration below which 90% of the isolate MIC values lie.

trations ⩽12.5 μM, and four isolates (MRSA 14320, MRSA 1000718, MRSA 0916054 and MSSA 15760) exhibited a MIC of 25 μM. Only one *S. aureus* (MSSA 15760) isolate showed a higher MIC than the other strains (with H2-74, MIC=100 μM) (Table 3). There were no obvious differences between the $MIC_{50}$ values of the four derivatives, but when comparing the $MIC_{90}$ values, the anti-staphylococcal activities of H2-38 and H2-81 were greater than those of H2-39 and H2-74 (Table 2). H2-38 and H2-81 were used for further evaluating the anti-biofilm efficacy of the derivatives against the clinical staphylococcal strains.

### Anti-biofilm efficacy against clinical staphylococcal strains

The microtiter plate assay showed that the minimal biofilm eradication concentrations of H2-38 and H2-81 against immature (6 h) biofilms of *S. epidermidis* ATCC 35984 were 12.5 μM and 6.3 μM, respectively. At a concentration of 6.25 μM, immature biofilms of all of the tested isolates (eight MRSE, four MSSE, five MRSA and two MSSA) were inhibited by both derivatives.

**Table 3 The MIC distribution for the four derivatives and the clinical staphylococcal isolates**

| | MIC[a] | | | | | |
|---|---|---|---|---|---|---|
| | ⩽12.5 (μM) | | 25–50 (μM) | | >50 (μM) | |
| Derivatives | SE[b] | SA[b] | SE | SA | SE | SA |
| H2-38 | 68 | 95 | 0 | 0 | 0 | 0 |
| H2-39 | 68 | 91 | 0 | 4 | 0 | 0 |
| H2-74 | 67 | 91 | 1 | 3 | 0 | 1 |
| H2-81 | 68 | 95 | 0 | 0 | 0 | 0 |

[a] MIC represents the minimal inhibitory concentration of the derivatives against the test strains as determined by broth micro-dilution testing on a microtiter plate according to the standards of the American Clinical and Laboratory Standards Institute.
[b] SE represents *S. epidermidis* (68 clinical stains) and SA represents *S. aureus* (95 clinical strains).

Confocal microscopy with Live/Dead staining showed that at a concentration of 4×MIC (6.25 μM) both H2-81 and H2-38 had bactericidal activity against mature (24 h) biofilms of all six strong biofilm-forming clinical strains (two MRSA, one MSSA, two MRSE and one MSSE). The number of viable cells in the biofilm was determined by ImageJ software. With H2-81 treatment, the percentages of viable cells in the biofilms of the six strains were reduced to 1.6%–11.6% (Table 4 and Figure 2). With H2-38 treatment, the viable cells were decreased to 3.5%–16.2% (Table 4). For the controls, 75.4%–80.8% of the cells were viable in the biofilm treated with vancomycin (128 μg/mL), and 69.8% of the cells were viable in the biofilm treated with the prototype of Compound 2. Using 0.1% dimethyl sulfoxide (DMSO) as a negative control, 92.4% of the cells were viable in the biofilms.

## DISCUSSION

The increasing prevalence of antibiotic-resistant bacterial infections has become one of the greatest threats to human health. The clinical significance of staphylococci is closely related to their ability to form biofilms because biofilms are phenotypic resistance to antibiotics and often result in long-lasting infections.[30] Most antibiotics are active against planktonic bacteria but not against biofilms, and they fail to cure biofilm-associated infections.[31] The challenge of developing therapeutics to treat staphylococcal biofilm infections is that several characteristics of biofilms promote their antibiotic resistance, including the slow growth rate of bacteria in a biofilm, the high bacterial density and the binding of antibiotics to the biofilm slime.[32]

New types of antibiotics are urgently needed to combat the growing threat from MRSA and biofilm-forming strains that are resistant to

**Table 4 The percentages of viable cells in the biofilms of the six strains treated with H2-81 and H2-38**

| | | Strains[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | Treatment[a] | MRSA 1000234 | MRSA 916054 | MSSA 1815 | MRSE 1020081 | MRSE 915296 | MSSE 914111 |
| Viable cells/total (%) | H2-81 | 1.6 | 7.8 | 8.2 | 11.6 | 7.9 | 8.9 |
| | H2-38 | 3.5 | 10.4 | 7.5 | 16.2 | 8.6 | 12.3 |
| | Vancomycin | 80.1 | 75.6 | 77.8 | 75.4 | 80.8 | 79.6 |
| | DMSO[b] | 92.4 | 89.1 | 88.5 | 91.8 | 91.4 | 93.6 |
| MIC (μM) | H2-81 | 1.5 | 12.5 | 1.5 | 3.1 | 3.1 | 1.5 |
| | H2-38 | 1.5 | 12.5 | 1.5 | 6.3 | 3.1 | 1.5 |

[a] The six strains were selected randomly from 19 strong biofilm producers (MRSA 1000234, MRSA 916054, MSSA 1815, MRSE 1020081, MRSE 915296 and MSSE 914111).
[b] Stock solutions of the compounds were prepared in 0.1% DMSO. Biofilms treated with 0.1% DMSO were used as the negative control.

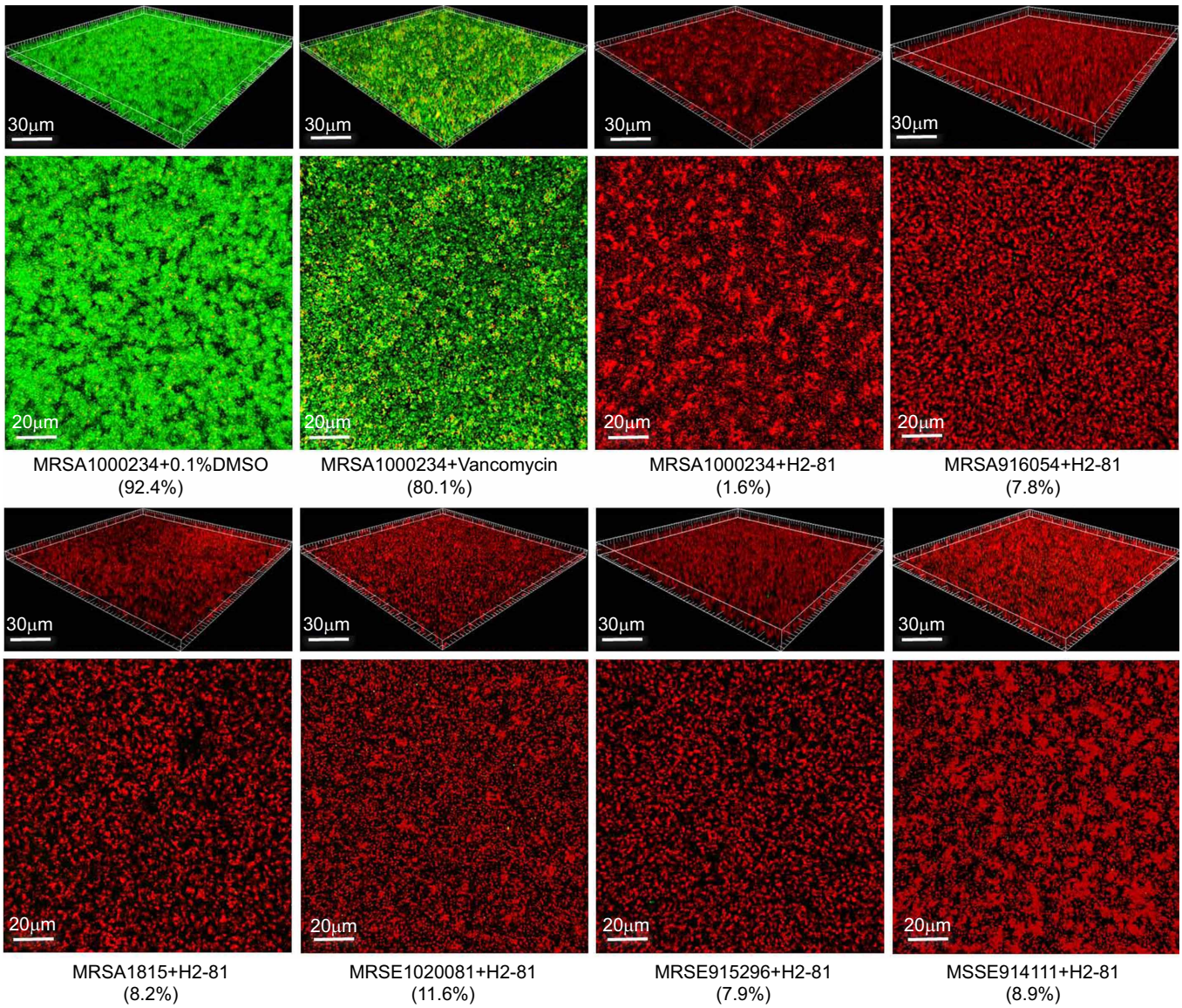


**Figure 2** The bactericidal activities of H2-81 on 24-h biofilms of the clinical staphylococcal strains. The six strong biofilm-forming clinical strains were incubated in Fluoro Dishes at 37 ℃ for 24 h, and the biofilms were treated with H2-81 at concentrations of 4×MIC (6.25 μM) for 12 h. The biofilms were visualized by confocal laser scanning microscopy with the Live/Dead viability stain (SYTO9/PI). In the three-dimensional structural images of biofilms, the viable cells exhibit green fluorescence, whereas dead cells exhibit red fluorescence. The percentages of live bacteria of the total bacterial counts as shown in brackets were calculated by ImageJ software.

conventional antibiotics.[33,34] The YycFG TCS and its orthologs (renamed as WalK/WalR)[35] are found in most low G+C gram-positive bacteria and play important roles in bacterial murein biosynthesis, cell wall metabolism and biofilm formation. TCSs have been regarded as potential targets for the development of new antibiotics, and inhibitors targeting YycG and YycF have been described. We previously reported that Compound 2, which targets the HK domain of the *S. epidermidis* YycG protein, inhibits *S. epidermidis* growth and biofilm formation.[20] The structure of Compound 2 was optimized by retaining the intact thiazolidione core structure and altering the functional groups. A total of 91 derivatives were synthesized. Four of these derivatives (H2-38, H2-39, H2-74 and H2-81) exhibited high antibacterial activity and were selected to evaluate their efficacy against clinical staphylococcus strains.

In the present study, the 163 staphylococcal isolates were collected from three hospitals as follows: 99 (60.7%) isolates were methicillin-resistant (MRSA and MRSE); 106 isolates (65.0%) were resistant to erythromycin; 55 isolates (33.7%) were resistant to SMZ-TMP; 68 isolates (41.7%) were resistant to gentamycin; and 27 isolates (16.5%) were resistant to rifampicin. A total of 43.1% of *S. aureus* isolates and 61.7% of *S. epidermidis* isolates were biofilm producers. We evaluated the efficacy of the derivatives against the clinical strains. The four derivatives inhibited the planktonic growth of all isolates, including the MRSA and MRSE isolates. These derivatives displayed bactericidal activities against both immature (6 h) biofilms and mature (24 h) biofilms produced by the strong biofilm-forming strains, thus demonstrating that they can act at both the early and late stages of biofilm formation.

Combinations of antibiotics can enhance the effect of individual antimicrobials through synergic interactions and are considered to be a new therapeutic option for the treatment of staphylococcal biofilm-associated infections.[36] However, it is important to choose the correct antibiotics for combination therapy. Rifampin is currently the most common constituent of antibiotic combinations against staphylococcal biofilms, and other frequently used antimicrobials are vancomycin and fusidic acid.[32,37] In a preceding study, we demonstrated a synergistic effect between two of the derivatives (H2-74 and H2-81) and vancomycin or cefazolin on the *S. epidermidis* 3 5984 strain,[23] thereby suggesting that these derivatives can be used alone or in combination with other antibiotics to combat staphylococcal biofilms.

In summary, the newly synthesized derivatives of Compound 2 exhibit substantial anti-staphylococcal activity against both methicillin-susceptible and methicillin-resistant clinical strains. Thus, these results warrant further investigation to develop a YycG inhibitor as a candidate drug that will be effective against multidrug-resistant staphylococci infections and biofilm-associated diseases.

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