The Antibacterial and Anti-biofilm Activities of Two Thiazolidione Derivatives (H2-60 and H2-81) Against Clinical *Enterococcus Faecium* Strains

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Abstract

Background: Previous reports have demonstrated two thiazolidione derivatives (H2-60 and H2-81) can robustly inhibit the planktonic growth and biofilm formation of *S. epidermidis* and *S. aureus* by targeting the histidine kinase (HK) YycG**.** Whereas the antibacterial and anti-biofilm activity of these two thiazolidione derivatives (H2-60 and H2-81) against *Enterococcus faecium* remains elusive. Here, the YycG recombinant proteins containing HisKA and HATPase_c domain of *E. faecium* DO were *in vitro* expressed in *E. coli* competent cell BL21 (DE3) and then purified for the autophosphorylation test, indicating these two thiazolidione derivatives (H2-60 and H2-81) could directly impact the kinase phosphoration activity of YycG of *E. faecium*.

Results: The MICs of H2-60 and H2-81 in the clinical isolates of *E. faecium* was in the range from 3.125mg/L to 25mg/L. Moreover, either H2-60 or H2-81showed the excellent bactericidal activity against *E. faecium* with the single dose or its combination with daptomycin (4 × MIC) by time-killing assay. Furthermore, over 90% of *E. faecium* biofilm formation could markedly be inhibited by two thiazolidione derivatives (H2-60 and H2-81) within 1/4×MIC value. In addition, the frequency of the eradicated viable cells embedded in mature biofilm were evaluated by the confocal laser microscopy, suggesting that of H2-60 combined with ampicillin or daptomycin was significantly high when compared with its monotherapy (78.17% and 74.48%*vs.*41.59%, respectively, $P< 0.01$).

Conclusion: Two thiazolidione derivatives (H2-60 and H2-81) exhibit the robust antibacterial and anti-biofilm activity against *E. faecium* by targeting the histidine kinase (HK) YycG.

Background

Enterococcus spp. is known to be capable of causing nosocomial and life-threatening infections in human. *Enterococcus faecium* infection can result in a variety of infectious diseases, including intra-abdominal and pelvic regions infections, urinary tract infections, implantable infections, biofilm-related infections *et al*. In recent years, the nosocomial infections caused by *E. faecium* has increasingly reported worldwide and the majority of clinical *E. faecium* isolates showed the capacity for the biofilm formation, which could often enhance the difficulty of antimicrobial treatment to suppress the protracted and chronic infections [1–3]. The biofilm formation limits the diffusion and penetration of bacteria due to the aggregation of extracellular polymeric substances (EPS) matrix. One of the most difficulties for the treatment of biofilm-related infection is to eradicate established biofilms on the surfaces of devices or cavities [9–10]. Recently, the increasing reports of multidrug resistant *E. faecium* strains, including vancomycin-,daptomycin- or linezolid-resistant strains, has aroused our attention. Therefore, the development of novel antimicrobial agents against the planktonic growth and biofilm formation of *E. faecium* would be beneficial for clinicians to improve the prognosis of these infections.

Previous studies have confirmed the decisive role of two-component systems (TCSs) of bacteria in the planktonic growth and biofilm formation of a variety of bacteria [4, 5]. Therefore, TCSs have aroused the

increasing attention as potential targets for the development of antibacterial and anti-biofilm drugs. YycFG is one of the TCSs involved in biofilm formation, cell wall metabolism, resistance and regulation of virulence factors in *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus* [6–9]. The histidine kinase YycG is a transmembrane protein and the kinase ATP-binding domain (HATPase_c domain) can sense external signals and is activated by extracellular signals, which subsequently resulting in the phosphorylation of the histidine site (HisKA domain)of YycG. By targeting the autophosphorylation of YycG TCSs, small molecule compounds with antibacterial activity have been effectively developed against the gram-positive bacteria such as *B. subtilis*, *S. aureus*[10, 11], *S. epidermidis* and *Streptococcus*[12]. Our group previously described the bactericidal and anti-biofilm activities of two YycG inhibitors, H2-60 and H2-81, by targeting the HATPase_c domain of *S. epidermidis* YycG [13], *S. aureus* [14]and *E. faecalis* (unpublished data).Therefore, YycG may be a potential alternative target for the development of antimicrobial agents and it need to be studied that whether the two YycG inhibitors (H2-60 and H2-81)are capable to block the planktonic growth and biofilm formation in *E. faecium*.

In this study, the interaction of two YycG inhibitors of the thiazolidione derivatives (H2-60 and H2-81) with the kinase ATP-binding site (HATPase_c domain) of YycG was investigated and the antibacterial and anti-biofilm activities of these two chemicals against *E. faecium* have be studied. Moreover, the potential application prospects of the combination therapy of two thiazolidione derivatives (H2-60 and H2-81) with the common antibiotics on biofilm formation of *E. faecium* were also assessed.

Results

The inhibitory effect of two thiazolidione derivatives on the Kinase activity of the recombinant E. faecium YycG'

In order to verify the impact of two thiazolidione derivatives on the kinase activity of the recombinant YycG' proteins of *E. faeciumin in vitro*, Kinase-Glo® Luminescent Kinase (Promega) was adopted to detect the correlation between relative light unit (RLU) and ATP content in the reaction system. Our data indicated the close correlation between the ATP concentration and the RLU (R^2 = 0.9907, Fig. 1A). Moreover, the kinase activity of the recombinant protein YycG' were also verified *in vitro*, suggesting the optimized amount of ATP was shown in Fig. 1B and3 μg YycG' protein were suitable for the addition with the various concentration gradients of ATP (1.56-50 μM) for the further detection of the kinase activity. RLU value decreased significantly in comparison to the control group without the addition of YycG' protein in addition of ATP concentration between 3.13–6.25 μM. Based on the above results, the effect of two thiazolidione derivatives on the kinase activity of YycG were detected with the concentration of 4 μM ATP and 3 μg recombinant protein suggesting the concentration-dependent inhibition of the activity of YycG' protein kinase by H2-60 andH2-81 respectively. The IC_{50} values (the concentration of compound inhibiting 50% of YycG' kinase activity) of H2-60 and H2-81 respectively were in the range of 25.4–26.2μM (Table 1).

Table 1
Biological activities of the two YycG inhibitors

Compounds	Molecular formula	MW	MIC[a] (μM)	MBC[a] (μM)	IC_{50}[b] (μM)
H2-60	$C_{27}H_{16}F_2N_2O_3S_2$	518	3.13	12.5	25.4
H2-81	$C_{27}H_{17}FN_2O_3S_2$	500	3.13	12.5	26.2
MW, molecular weight; [a] MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) against *E. faecium* EF16M64.					
[b]IC_{50} represents the concentration of the YycG in inhibitors that inhibited 50 percent of the autophosphorylation of YycG' detected by Kinase-Glo Luminescent kit.					

Antibacterial activity of two thiazolidione derivatives (H2-60 and H2-81) against E. faecium

The antibacterial activities of H2-60 and H2-81 against 102non-repetitive clinical *E. faecium* strains were determined by broth microdilution method, suggesting the MIC values of these two chemicals ranging from 3.13μM to 25 μM. Moreover, the MIC_{50}/MIC_{90} values of these clinical *E. faecium* strains were 6.25/6.25μM for H2-60and 6.25/12.5μM for H2-81 respectively (Table 2). The MIC values of ampicillin, vancomycin, linezolid and daptomycin against *E. faecium* EF16M64 were listed in Supplementary Table S1.The MIC value and the MBC value for *E. faecium* EF16M64 was 3.13μM and12.5μM respectively. The effect of H2-60 and H2-81 on planktonic bacterial growth at different sub-inhibitory concentrations were evaluated, suggesting the unaffected growth of planktonic bacteria by these two chemicals at the concentration of 1/4 × MIC or below (Table 1 and Fig. 2).

Table 2
MIC values of YycG inhibitors against 102clinical *E. faecium* strains

Compounds	No of the MIC values creep				MIC_{50}	MIC_{90}
	3.13	6.25	12.5	25	(μM)	(μM)
H2-60	2	79	17	4	6.25	6.25
H2-81	2	48	47	5	12.5	12.5

Time-killing assay of two thiazolidione derivatives (H2-60 and H2-81) against E. faecium

Time-killing assay were performed with two thiazolidione derivatives (H2-60 and H2-81) alone or combined with ampicillin, vancomycin, linezolid, or daptomycin at concentrations of 4× MIC respectively. Within 24 h, the amount of the viable bacterial cells can be decreased with single dose of H2-60 or H2-81by more than 2×$\log_{10}$CFU counts (Log_{10}CFU/mL) in comparison to that treated with daptomycin or linezolid. Worthy of our attention, when H2-60 orH2-81 combined with daptomycin, the amount of viable bacteria cells could be decreased by more than 3×$\log_{10}$CFU within 11 h and the bactericidal effect of the combination would sustain over 24 h with the of the viable bacterial cells reduced to the amount of less than 10 CFU (Fig. 3).

Anti-biofilm activity of two thiazolidione derivatives (H2-60 and H2-81) against E. faecium

The biofilm formation of 102 *E. faecium* strains from different sources was assayed using polystyrene microtiter plate. EF16M2 strain($OD_{570} \approx 0.207 \pm 0.03$)was regarded as negative control and OD_{570} cut-off (OD_c) value was calculated to be 0.297. 52.9% (54/102) of the clinical *E. faecium* strains was shown positively with biofilm formation, 7.8% (8/102) were shown with strong positive biofilm phenotype, 11.8% (12/102)were medium positive biofilm phenotype, and 33.3%(34/102) could form the weak positive biofilm (Table 3).

Table 3
Occurrence of *E. faecium* biofilm formation by clinical source

Clinical source (no. isolates tested)	No. (%) of isolates with biofilm phenotype				
	Strong	medium	Strong or medium	Weak	All positive
Blood (9)	1 (11.1)	1 (11.1)	2 (22.2)	3 (33.3)	5 (55.6)
Urine (55)	3 (5.5)	7 (12.7)	10 (18.2)	19 (34.5)	29 (52.7)
Pus or secretions (10)	2 (20.0)	0 (0.0)	2 (20.0)	3 (30.0)	5 (50.0)
Bile (6)	1 (16.7)	1 (16.7)	2 (33.3)	2 (33.3)	4 (66.7)
Other[a] (22)	1 (4.5)	3 (13.6)	4 (18.2)	7 (31.8)	11 (50.0)
Total (102)	8 (7.8)	12 (11.8)	20 (19.6)	34 (33.3)	54 (52.9)

[a] Other sources contained sputum, ear swab, feces, catheter, pleural effusion, ascites fluid, drainage fluid and bronchoalveolar lavage fluid.

Our data have demonstrated the unaffected planktonic growth of *E. faecium* by two thiazolidione derivatives (H2-60 and H2-81) at 1/4×MIC concentration(shown in Fig. 2).8 strains of *E. faecium* with the range of positive biofilm formationOD_{570} from 1.27 to 2.814were selected to evaluate the inhibitory effect of H2-60 and H2-81at sub-MIC concentration (1/4 × MIC), indicating that the significantly reduced biofilm

formation when treated with 1/4 ×MIC of H2-60 or H2-81for 24 h respectively($P < 0.0001$) (shown in Fig. 4).

Eradication of mature biofilm of E. faecium by two thiazolidione derivatives (H2-60 and H2-81) using CLSM

CLSM were performed for the quantification of the amount of the viable bacteria cells, suggesting the amount of viable biofilm-embedded bacteria cells in the mature biofilm were reduced to 41.6% and 29.0%by H2-60 and H2-81 at 3.13μM (1× MIC) respectively. Moreover, 78.17% and 74.48% of viable biofilm-embedded bacteria cells in mature biofilms could be eradicated by H2-60 combined with 8× MIC of ampicillin or daptomycin in comparison to the monotherapy with H2-60 (Fig. 5 and Table 4).

Table 4
The proportion of viable cells or dead cells within mature biofilm using ImageJ software

Compounds	SYTO9	PI	PI/Total
H2-60	19.785±25.843	12.374±18.152	41.59%
H2-81	24.691±25.063	10.065±25.322	28.96%
AMP	41.525±49.978	13.659±26.067	24.75%
DAP	28.258±28.01	27.802±30.489	49.6%
H2-60 + AMP	13.905±22.923	49.795±53.822	78.17%
H2-60 + DAP	18.878±25.598	55.093±64.402	74.48%
H2-81 + AMP	37.991±29.629	8.66±20.861	18.56%
H2-81 + DAP	67.603±55.643	36.008±51.793	34.75%
DMSO	131.508±57.004	2.565±10.567	2.0%

Discussions

The rapid emergence of multidrug-resistant *Enterococcus spp*. has limited the available drug options for the treatment of *Enterococcus* infections in human. Currently, just few antimicrobial agents are very effective for the treatment of biofilm-associated infections such as endocarditis, intravascular catheter-related infections, et al. Therefore, it's urgent for us to develop the effective compounds for the improvement of the clinical outcome of the biofilm-associated infections caused by *Enterococcus spp*. Previous reports have indicated the robust antibacterial and anti-biofilm activities of two thiazolidione derivatives(H2-60 and H2-81) against *S. epidermidis*, *S. aureus* and *E. faecalis* by targeting the histidine

kinase YycG of *S. epidermidis* without the cytotoxicity [14, 17, 18].The bioinformatic analysis data showed the highly homologous characteristics of the YycG-encoded protein of *E. faecium* compared with that in *S. epidermidis* and *S. aureus* (Supplementary Table S2). We hypothesize that two thiazolidione derivatives might target at the YycG of a broad-spectrum of gram-positive bacteria and exhibit both the antibacterial and anti-biofilm activity against these bacteria. In this study, our data supported the binding affinity of two thiazolidione derivatives (H2-60 and H2-81) with the recombinant YycG 'protein of *E. faecium*, suggesting the same target sites of H2-60 and H2-81 in *S. epidermidis* with that in *E. faecium*.

Here, our data showed the low MIC values of two thiazolidione derivatives (H2-60 and H2-81) against clinical *E. faecium* isolates. Time-kill assay further indicated the bactericidal activity of two thiazolidione derivatives (H2-60 and H2-81) similar to that with ampicillin, vancomycin, and linezolid at 4 × MIC concentration. In addition, both H2-60 and H2-81 could still maintain the bactericidal effect for over 24 hours when combined with daptomycin, suggesting the possible synergetic role of these two thiazolidione derivatives with daptomycin. Daptomycin is a lipopeptide antibiotic can block the peptidoglycan biosynthesis of the bacterial cell wall by disturbing the transport of amino acids of the cell membrane [19]. Multiple reports have demonstrated the involvement of YycG in the degradation of *S. aureus* cell wall by hydrolyzing peptidoglycan, and nucleotide insertion mutation in the *yycG* gene participate in the reduced daptomycin susceptibility in *S. aureus* [20]. Therefore, we speculated that these two thiazolidione derivatives can exert a synergistic bactericidal role with daptomycin by interfering with the synthesis of bacterial cell wall through binding YycG. Their possible synergetic mechanism of these two thiazolidione derivatives with daptomycin needs to be further elucidated.

Biofilm matrix is often composed of polysaccharides, extracellular DNA (EDNA), a variety of lipids and proteins *et al*. It's difficult to kill the viable bacteria cells that embedded in biofilm from the clinical environment. Currently, limited effective antibiotic can be used for the treatment of biofilm associated infections and the eradication of mature biofilms still remains an urgent clinical problem. Here, our data demonstrated the significant inhibitory effect of H2-60 and H2-81 on the *E. faecium* biofilm formation and the viable bacteria embedded in biofilm detected by CLSM can be eradicated by the single dose of H2-60. Furthermore, the combination of H2-60 with ampicillin or daptomycin could enhance the clearance capacity of the viable bacteria embedded in the mature biofilms. Therefore, H2-60 might be used as an adjunct to antibiotics with potential applications for the treatment of biofilm-associated infections caused by *E. faecium*.

Conclusion

Two thiazolidione derivatives (H2-60 and H2-81) targeting the HATPase_c domain of *S. epidermidis* YycG can also bind with the YycG of *E. faecium*. The MICs values of these two thiazolidione derivatives (H2-60 and H2-81) against *E. faecium* were similar to that of *S. epidermidis* previously reported [14]. Time kill assay demonstrated the synergetic bactericidal activity of two thiazolidione derivatives (H2-60 and H2-81) toward the planktonic growth of *E. faecium*. Moreover, the robust inhibition of these two chemicals on the biofilm formation of *E. faecium* were found and especially worthy of our attention, the combination

therapy of H2-60 with ampicillin or daptomycin could enhance the bactericidal activity toward the viable bacteria embedded in the *E. faecium* biofilm. Conclusively, two thiazolidione derivatives (H2-60 and H2-81) targeting the HATPase_c domain of *S. epidermidis* YycG have shown the antibacterial and anti-biofilm activity against *E. faecium*. It still needs to be studied that two thiazolidione derivatives might target at the YycG and exhibit both the antibacterial and anti-biofilm activity against a broad-spectrum of gram-positive bacteria.

Methods

Bacteria strains and materials

102 nonredundant clinical isolates of *E. faecium* were obtained from Huazhong University of Science and Technology Union Shenzhen Hospital. The common-used antibiotics, including vancomycin (catalog no. V2002), linezolid (catalog no. PZ0014), ampicillin (catalog no. A9518) and daptomycin (catalog no. SBR 00014), were purchased from Sigma Aldrich (Shanghai, China). Two YycG inhibitors of the thiazolidione derivatives (H2-60 and H2-81) were synthesized and obtained from WuXiAppTec (Shanghai, China). MHB, CAMH, TSB and Agar were purchased from Oxoid Ltd (Basingstoke, England). *E. faecalis* ATCC29212 (biofilm negative) were used as quality control for MIC determination and *E. faecium* EF16M64 were used as biofilm positive strains.

Antimicrobial susceptibility testing and MBC Assays

The *E. faecium* strains were cultured overnight on blood agar plates and these isolates were identified by the Phoenix 100 automated microbiology system (BD, Franklin Lakes, New Jersey, USA). The MIC values of two thiazolidione derivatives (H2-60 and H2-81), vancomycin, linezolid, ampicillin and daptomycin against *E. faecium* were determined by broth microdilution method according to the standards of Clinical and Laboratory Standards Institute (CLSI) in 2019. Briefly to describe the MICs detection of H2-60 and H2-81, two-fold serial dilutions of the compounds at final concentrations from 200 to 0.39 μM were prepared in 96-well microtiter plates (Falcon) containing 100 μL CAMHB medium. The medium was adjusted by sterile saline until the bacterial suspension density was equal to a 0.5 McFarland standard ($\sim 1.0 \times 10^8$ CFU/mL) and then diluted to 1:100 into CA-MHB medium. The diluted bacteria were added into 96 well plate (50 μL/well) and mixed and placed in 37℃ incubator and incubated at 220 rpm for 20-24h. The interpretation of MIC was the lowest concentration without visible cells growth in the well. 100 μL of bacteria solution with or above MIC value was coated on a new MHA plate and incubated at 37℃ for 24h for counting. The MBC value was represented as a 99.9% reduction of the original inoculum or the number of clones on each plate is ≤ 5. Besides, positive control (without compound) and negative control (without bacterial solution) were also set and *E.faecalis*ATCC29212 was used as quality control. MIC_{50}/MIC_{90} was used to represent MIC value distribution of all the clinical strains. All experiments were performed in triplicate.

Cloning, expression and purification of the YycG' protein

The YycG' fragment containing the cytoplasmic catalytic and ATP-binding domains (the HisKA and HATPase_c domain, see Fig. 1A) of YycG (377aa to 604aa) in *E. faecium* was amplified by PCR with the template of genomic DNA of *E. faecium* DO (Genbank No. NC_017960). The amplified primers were 5'-TTTGGATCCCGTCGTGAATTCGTCTCTAA -3' and 5'-TTTCTCGAGTAGGTTCATATGGCAGCGAGAT-3' respectively. Subsequently, the fragment was digested with *BamHI* and *XhoI* (Thermo Fisher Scientific, Massachusetts, USA) and ligated into the corresponding sites of pET28 (a) to obtain pET28 (a)-YycG'. After being transformed into *E. coli* strain BL21 (DE3), this recombinant plasmid was induced to express YycG' protein by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 22℃ for 12 h. Then, the bacterial cells were disrupted by sonication and centrifuged, the supernatant was purified by Ni–NTA agarose (Qiagen, Los Angeles, CA, USA) using affinity chromatography method. The concentration of recombinant YycG' protein was determined by the BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA), according to the manufacturer's protocol.

Inhibition Assay for phosphorylation activity of the HisKA domain of YycG'.

In order to verity interaction of two thiazolidione derivatives (H2-60 and H2-81) with the HisKA domain of YycG, impact of these two chemicals on the ATPase activity of the YycG' protein were measured using the Kinase-Glo^{TM} Luminescent Kinase Assay (Promega, Madison, USA). Briefly, 3 μg purified YycG' protein was pre-incubated with a series of dilutions of the compounds in reaction buffer [40 mM Tris (pH 7.5), 20 mM $MgCl_2$ and 0.1 mg/ml BSA] at room temperature for 30 min, 4 μM ATP was added and incubated for 30 min at room temperature, then Kinase-Glo^{TM} Reagent was added to detect the remaining amount of ATP, and the results were reflected by luminescence intensity value (RLU). Meanwhile, YycG' protein with no addition of the compounds was used as the control and ATP only was used as a blank. The rate of inhibition of kinase phosphorylation (Rp) by the compounds was calculated by the following equation:

$$\mathrm{Rp} = \frac{\mathrm{RLU(HK'+compounds+ATP)-RLU(HK'+ATP)}}{\mathrm{RLU(ATP\ only)-RLU(HK'+ATP)}} \times 100\%$$

IC_{50} (the concentration resulting in 50% inhibition of YycG' histidine kinase auto-phosphorylation) was obtained by using GraphPad v7.0 software (San Diego, CA, USA).All experiments were performed in triplicate.

Growth curves of the planktonic bacteria

After the overnight incubation, liquid cultures of *E. faecium* EF16M64 were sub-cultured (1:200) in fresh TSB medium and two thiazolidione derivatives (H2-60 and H2-81) were doubling diluted (from 1×MIC to 1/16×MIC) by TSB medium. After diluted, totally300μL dilution were added to growth curve device (Bioscreen C co., Piscataway, USA) in triplicate. The bacterial solution without the derivatives was used as positive control and the effect of the derivatives on bacterial growth was automatically monitored every hour by measuring the optical density at 600 nm (OD_{600}) continuously for 16 hours. The experiments were performed in triplicate.

Time-kill assay

Time-kill curve of single dose of two inhibitors and combination with vancomycin, linezolid, ampicillin, or daptomycin at concentrations of 4× MIC respectively were assayed with the suspensions of *E. faecium* EF16M64(~1×10^6 CFU/mL). Time-kill experiments were performed at 37°C with shaking at 220 rpm under aerobic conditions according to previously reported [15] Aliquots (1 mL) were removed at different time points (0, 1, 3, 5, 7, 11, and 24 h), serially diluted with sterile saline, and 100 μL of bacteria were evenly coated on MHA plate and cultured at 37℃ for 24h, bacterial colonies were counted and determined by plotting $\log_{10}$ colony counts (CFU/mL) against time. Bacteria treated with 0.1% DMSO served as a control. The results were independently presented as the mean ± standard deviations (SD) in triplicate.

Determination of *E. faecium* biofilm formation

Biofilm formation assays were performed by using the methods described in our previous study [16]. Briefly, overnight cultures of *E. faecium* EF16M64 were diluted 1:200 with fresh TSBG (TSB plus 0.25% glucose) and inoculated into 96-well polystyrene microtiter plates (200μL/well,Costar3599; Corning). After incubation at 37℃ for 24 h, the supernatant of the unattached cells was removed and washed with deionized water for three times, adherent biofilms in the plates were fixed with 95% methanol, stained with 1% crystal violet for 20 min and washed, and then dried in air for 2 h. The optical density at 570 nm (OD_{570}) was tested by micro plate spectrophotometer (DTX880, Beckman Coulter, USA). *E. faecium* strains EF16M64 and EF16M2 were used as the biofilm-positive and -negative controls, respectively. According to the literature [14], the biofilm formation phenotypes were divided into four categories. The cut-off value of OD_c was defined as three standard deviations above the mean OD of the negative control. The classification is as follows: $OD_{570}\leq OD_c$, no biofilm formation, $OD_c< OD_{570}\leq 2\times OD_c$, weak biofilm formation, $2\times OD_c< OD_{570}\leq 4\times OD_c$, medium biofilm formation, $OD_{570}> 4\times OD_c$, strong biofilm formation.

Anti-biofilm activity of two thiazolidione derivatives with sub-MIC concentration

The inhibitory effect of two inhibitors (H2-60 and H2-81) against the biofilms formation of *E. faecium* for 24 h was observed in TSBG using a semi-quantitative assay. An overnight bacteria culture was diluted 1:200 into TSBG. Aliquots of the inhibitors at 1/2×MIC concentrations were mixed with the same volume of the bacterial cultures in TSBG, added to 96-well polystyrene plates in triplicate, and co-cultured under static conditions for 24 h. After washing with sterile saline, the biofilms were stained with crystal violet and the OD_{570} was measured. Wells with no derivatives were used as positive control. All the experiments were repeated in triplicate, and the data represent the means ± standard deviations (SD).

Determination of cell viability in mature biofilms by CLSM

The effect of two thiazolidione derivatives on cell viability in mature biofilms (24 h) was determined using the Live/Dead Bacterial Viability method (Live/Dead BacLight, Molecular Probes, USA) with SYTO 9 and propidium iodide (PI) dyes to stain live and dead cells within mature biofilms. Overnight cultures of *E.faecium*EF16M64 was grown in cell-culture dishes (Fluorodish, FD35-100)with TSBG medium and incubated at 37°Cfor 24 h. Planktonic bacteria were then removed and discarded, and fresh TSBG containing the inhibitors monotherapy (at MIC concentrations) or the inhibitors combined with a final concentration of 8× MIC of ampicillin and daptomycin were added and incubated at 37°Cfor a further 72 h, exchanging the media every 24 h for fresh media containing appropriate inhibitors. After staining, mature biofilms were observed under a CLSM (Leica) with oil-immersion objective. IMARIS 7.0.0 software (Bitplane) was used to edit and analyze the original images. Green and red fluorescence represented the viable and dead cells, respectively. The PI/total percentage, representing the proportion of dead cells within the mature biofilm, was estimated using ImageJ software (Rawak Software Inc., Stuttgart, Germany). This assay was performed in triplicate and similar results were obtained.

Statistical analysis

Experiments were performed in triplicate and repeated at least three times. Student's t-test was utilized for data comparison. Differences in means were considered significant when $P<0.05$.

Abbreviations

HK: histidine kinase; EPS: extracellular polymeric substances; TCSs: two-component systems; CLSM: confocal laser scanning microscope;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files]

Competing Interest

The authors declare that they have no competing interests.

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Authors’ contributions

ZC, YX, YT and YZ contributed equally to this work. ZC designed the study, performed bioinformatic analysis, inhibition assay and biofilm assay. YX conducted the cloning, expression and purification of the protein. YT and YZ conducted the mRNA extraction and qPCR data analysis. JC and JZ collected the samples, performed the antibacterial activities determination. YW performed the CLSM analysis. QD reviewed the manuscript. ZC, DQ and JZ designed the study, participated in the data analysis, and provided critical revisions of the manuscript for valuable intellectual content. All authors were involved in the design of this work.

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Figures

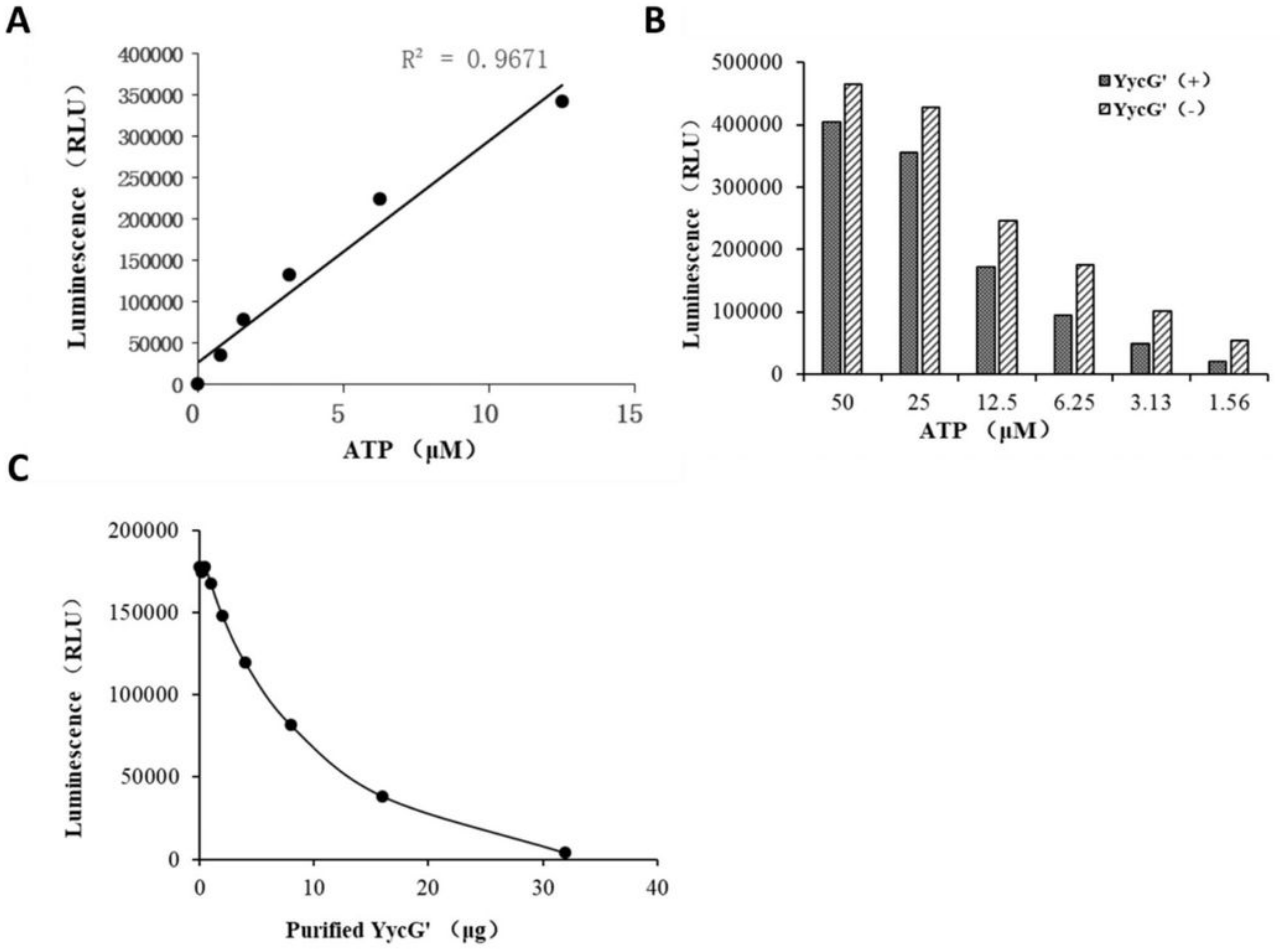

Fig. 1

Figure 1

Validation of the correlation ship between YycG' recombinant protein kinase and ATP consumption in vitro. Luminescence had a direct correlation with the consumption of ATP. (A) Data showed that a positive relationship between the RLU and the consumption of ATP with variant concentrations from 0-12.5μM measured with the Kinase-Glo® Assay (R2 = 0.9671). (B). 3 μg of YycG' recombinant protein was added into reaction system containing variant ATP concentrations (1.56~50μM), systems without YycG' present were used as control. (C). Variant amount of YycG' proteins (0.5~32 μg) were added into the systems with 4μM ATP. Data (means ± SDs) were performed in triplicate independently.

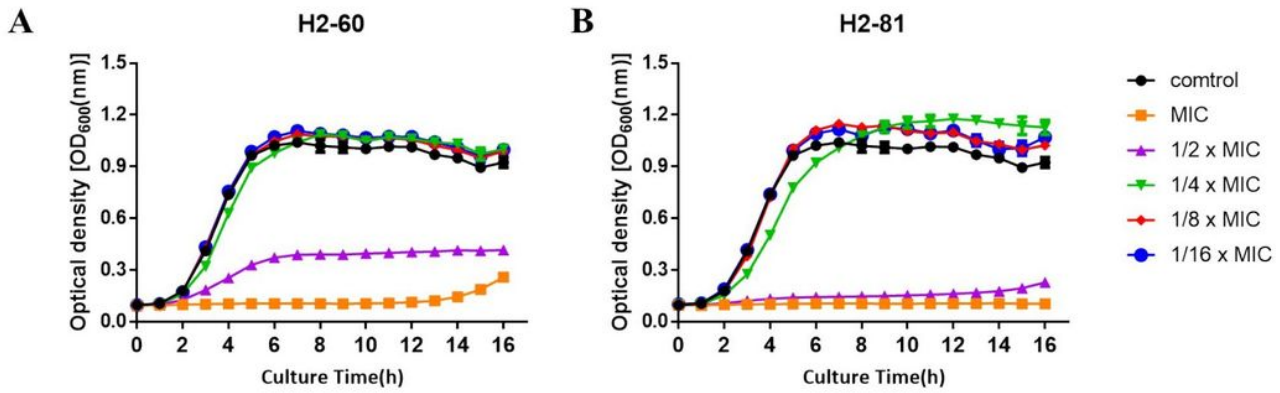

Fig. 2

Figure 2

Growth curves of YycG inhibitors against planktonic bacteria of E. faecium EF16M64 in sub-MIC concentrations. Overnight cultures of E. faecium EF16M64 strains were diluted 1:200 into 1 mL TSB. Aliquots of variant concentrations of inhibitors (from MIC to 1/16×MIC) were serially diluted and mixed with the culture medium, then totally 300 μL of the culture were added into 100-well plate. The plates were incubated in 37°C and monitored by measuring the OD600 using Bioscreen (Germany). Data (means ± SDs) were performed in triplicate independently. (A) Growth curve of H2-60 against planktonic bacteria of E. faeciumEF16M64. (B) Growth curve of H2-81 against planktonic bacteria of E. faeciumEF16M64.

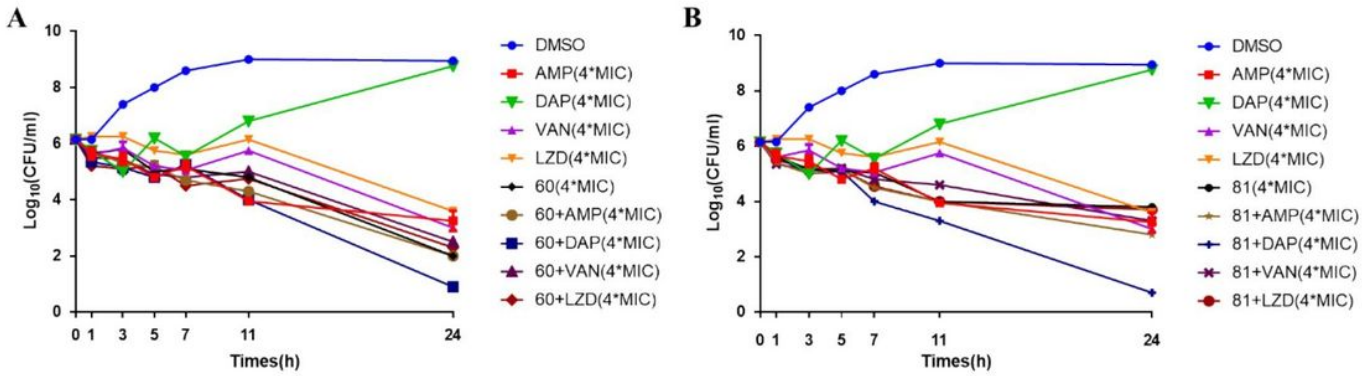

Fig. 3

Figure 3

Time-killing curve of YycG inhibitors combined with ampicillin, vancomycin, linezolid, or daptomycin against E. faecium EF16M64. Bacteria culture were respectively treated with YycG inhibitors alone or in combination with ampicillin, vancomycin, linezolid, or daptomycin at concentrations of 4× MIC value at

37℃ for 24 h. Aliquots (1 mL) were collected from the medium at 0, 1, 3, 5, 7, 11, and 24 h, serially diluted, and 100 μL of each dilution was spotted onto MHA plates and incubated at 37℃ for 16-24 h. Viable bacteria (CFU) were then counted. The activity of each compound was determined by the CFU counts (Log10CFU/mL) for each time points. Bacteria treated with 0.1% DMSO was used as a control. Results are presented as the mean ± SD from triplicate independent repeats.

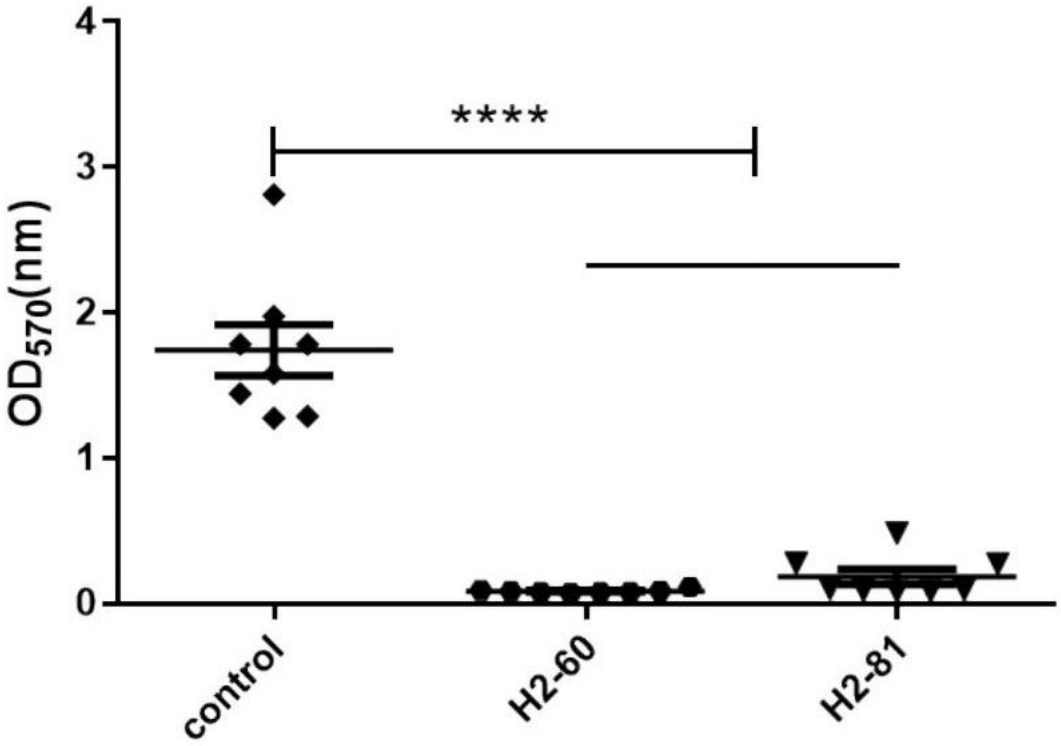

Fig. 4

Figure 4

Two YycG inhibitors inhibited the biofilm formation of E. faecium strains in sub-MIC concentration. Overnight culture of the E. faecium strains were diluted 1:200 with fresh TSBG (TSB plus 0.25% glucose), Aliquots of inhibitors with 1/4×MIC concentration were mixed with the TSBG, added to 96-well polystyrene plates in triplicate and co-cultured under static conditions for 24 h. After washing, the biofilms were stained with crystal violet. The plates were analyzed at OD570. The experiments were repeated in triplicate, each data represented as the average of the results of three independent experiments. ****: vs control group, P<0.0001.

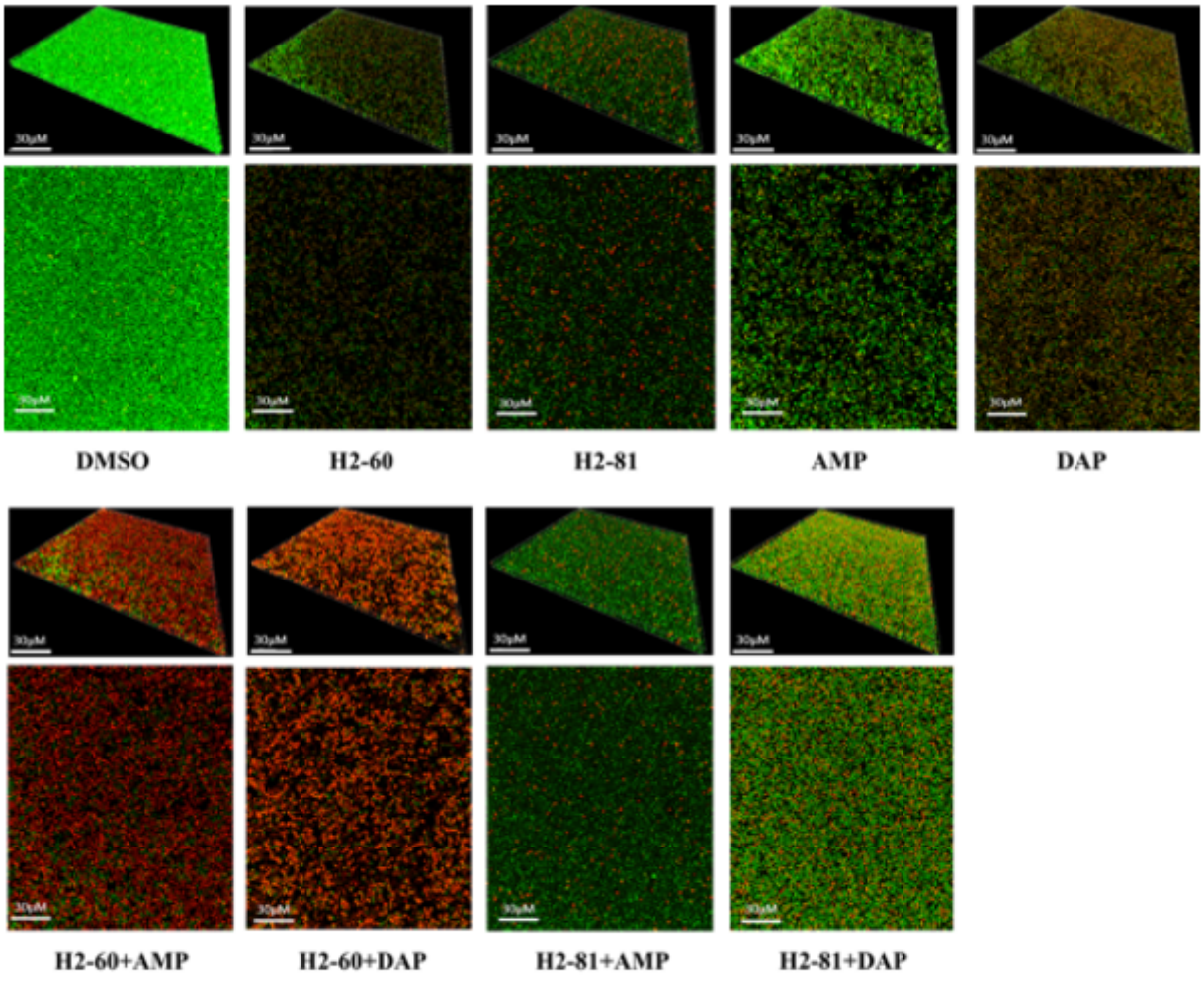

Fig. 5

Figure 5

Effects of the two YycG inhibitors on mature biofilm of E. faecium EF16M64. E. faecium EF16M64 was grown in cell-culture dishes with TSBG (TSB plus 0.25% glucose) medium and incubated at 37℃for 24 h, the planktonic bacteria were removed and fresh TSBG with MIC concentrations of inhibitors or combined with a final concentration of 8 ×MIC of ampicillin and daptomycin were added and incubated at 37for 72h, every 24h exchanging the liquid with the fresh concentration of compounds. SYTO 9 and propidium iodide (PI) dyes were stained with alive and dead cells within mature biofilms and was observed under a confocal laser scanning microscope (CLSM, Leica) with oil-immersion objective. IMARIS 7.0.0 software (Bitplane) was used to edit and analyze the original image. Green and red fluorescence were represented as the viable and dead cells respectively. The PI/total percentage represented as the proportion of dead cells within mature biofilm using ImageJ software. DAP: daptomycin; AMP: ampicillin.

Supplementary Files

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