The synergy of berberine chloride and totarol against *Staphylococcus aureus* grown in planktonic and biofilm cultures

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*Staphylococcus aureus* (*S. aureus*) is commonly associated with hospital-acquired infections and is known to form biofilms. Bacteria inside biofilms display an increased resistance to chemotherapeutics and host immune defences. Efficient antibiotics or combination therapy are urgently needed to treat patients with biofilm-associated MRSA infections. The objective of the current study was to evaluate the *in vitro* antimicrobial activities of totarol alone or in combination with berberine chloride (BBR) against *S. aureus* grown in planktonic and biofilm cultures. The synergistic antimicrobial effects between BBR and totarol were observed in all tested strains grown in biofilms using a chequerboard microdilution method, with the fractional inhibitory concentration index values ranging from 0.125 to 0.375. No antagonistic activity was observed in any of the strains tested in suspension or biofilm cultures. The synergistic activity against *S. aureus* biofilms was also corroborated by confocal laser scanning microscopy and adhesion assays. Moreover, the present study demonstrated that combination BBR and totarol treatment effectively decreased the formation of *S. aureus* biofilms by affecting extracellular genomic DNA release and polysaccharide intercellular adhesin expression. Subsequently, real-time reverse transcriptase PCR analysis revealed that the combination of BBR and totarol effectively inhibited the transcription of the biofilm-related genes *sarA*, *cidA* and *icaA*. These results suggest that the combination of totarol and BBR is momentous for the further development of a therapy protocol against *S. aureus* biofilms.

INTRODUCTION

*Staphylococcus aureus* (*S. aureus*) is one of the most significant pathogens in both hospitals and the community and can cause numerous syndromes in humans, such as endocarditis, pneumonia and septicaemia (Bubeck Wardenburg et al., 2007; Panizzi et al., 2011). More than 60 % of *S. aureus* isolates are resistant to meticillin, and the emergence and spread of meticillin-resistant *S. aureus* (MRSA) have become a worldwide challenge (Koziel et al., 2009).

Abbreviations: BBR, berberine chloride; eDNA, extracellular genomic DNA; EPI, efflux pump inhibitor; FICI, fractional inhibitory concentration index; MBBC, minimum biofilm bactericidal concentration; MBC, minimum bactericidal concentration; MBIC, minimal biofilm inhibition concentration; MDR, multi-drug resistance; MHA, Mueller–Hinton agar; MHB, Mueller–Hinton broth; MRSA, meticillin-resistant *S. aureus*; PIA, polysaccharide intercellular adhesion; TSB, Tryptic soy broth; WGA–HRP, wheatgerm agglutinin coupled to horseradish peroxidase.

Moreover, bacteria that form biofilms were reported to exhibit significantly increased resistance to antibacterials (Gilbert et al., 2002). Biofilms are defined as complex and organized bacterial communities that are embedded in a self-produced extracellular polymeric matrix and are rarely eliminated (Wang et al., 2009). Biofilms have been reported to possess 100–1000-fold reductions in susceptibilities towards antimicrobials compared to equivalent populations of their free-floating counterparts (Stewart & Franklin, 2008). It is urgently necessary to find effective combination therapy or novel drugs to overcome the problem of drug resistance in biofilm-associated *S. aureus*.

Based on this serious resistance problem, different methods to target drug-resistant *S. aureus* infection must be explored. One approach is to develop new antimicrobial agents, whereas another is to improve the efficacy of antimicrobial agent therapy for difficult infections using combination therapy (Nguyen & Graber, 2010). Indeed, certain
drug combinations have been empirically demonstrated to work synergistically (Amaral et al., 2004).

Berberine chloride (BBR), a protoberberine alkaloid extracted from *Coptidis rhizoma*, possesses antimicrobial activity against both Gram-positive and Gram-negative bacteria and other micro-organisms (Amin et al., 1969; Iwasa et al., 1998). BBR also exhibits antimalarial, anti-inflammatory, antisecretory and anticancer activities with relatively low cytotoxicity to human cells and has been used in clinical applications for many years (Chung et al., 1999). Recently, BBR has been used as a novel agent against a number of pathogenic micro-organisms, including bacteria and fungi (Hayashi et al., 2007; Yu et al., 2007; Park et al., 2006). Reports have indicated that BBR could effectively inhibit the formation of *S. epidermidis* biofilms (Wang et al., 2009).

Totarol, a diterpene compound isolated from the totarol tree, possesses effective activity against Gram-positive bacteria and extraordinary activity against *S. aureus* (Muroi & Kubo, 1996). One report showed that totarol was a potent efflux pump inhibitor (EPI) in *S. aureus* and could reduce biofilm formation (Smith et al., 2007). However, the synergistic effectiveness of BBR and totarol on antibacterial activity has not been reported.

The aim of this current investigation was to evaluate the antimicrobial efficacy of BBR alone and in combination with totarol against planktonic and biofilm cultures of *S. aureus*. This study might provide an alternative method to overcome the problem of *S. aureus* drug resistance both in suspension and in biofilms.

**METHODS**

**Bacterial strains and reagents.** The quality control strain ATCC 29213 was obtained from the China Medical Culture Collection Center (GMCC). Ten clinical meticillin-resistant *S. aureus* (MRSA) isolates were obtained from the Jilin Provincial People’s Hospital. Antibiotic resistance profiles of the ten MRSA isolates were tested against nine antibiotics in our previous experiments as recommended by the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2004, 2011). The isolates were resistant to penicillin (100%), trimethoprim/sulfamethoxazole (100%), erythromycin (90%), clindamycin (90%), doxycycline (80%), oxacillin (90%), chloramphenicol (80%), gentamicin (80%) and ciprofloxacin (20%). In contrast, all of the MRSA isolates were susceptible to vancomycin.

BBR and totarol were purchased from Sigma-Aldrich, and stock solutions were made in DMSO (Sigma-Aldrich). Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were purchased from BD Biosciences. Tryptic soy broth (TSB) was purchased from Oxoid.

**Growth conditions of the bacterial strains.** All tested strains were maintained at −80 °C in MHB containing 15% glycerol. Suspensions of each test strain were prepared following inoculation of MHB with an isolated colony from MHA and incubation for 24 h. For the biofilm-related inoculum preparation, an isolated colony was inoculated into TSB containing 0.25% glucose (TSB-g) and incubated at 37 °C for 24 h.

**Antimicrobial susceptibility testing for *S. aureus** in suspension.** The MICs of BBR and totarol against the *S. aureus* strains were determined by broth microdilution using twofold serial dilutions in MHB as described by CLSI (2009). The MIC was expressed as the lowest concentration showing no growth. The minimum bactericidal concentrations (MBCs) were determined by mixing the contents of each well (10 µl) with molten MHA broth. After setting, the plates were incubated at 37 °C for 24 h. The MBC point was defined as the lowest concentration showing no microbial growth. Each isolate was tested in triplicate on different days.

**Interactions between BBR and totarol against *S. aureus in suspension.** The interaction between BBR and totarol against 10 MRSA *S. aureus* strains and ATCC 29213 was tested using the microdilution checkerboard method (Odds, 2003). Serial twofold dilutions were prepared in MHB ranging from 1/512 to 4-fold the MIC concentration for BBR and from 1/64 to 4-fold the MIC for totarol. A total of 50 µl of each BBR concentration was added to columns 2–11, and then 50 µl of totarol was added to rows B–G. A total of 50 µl of MHB was added to column 1 and row A. The checkerboard plates were inoculated with 10^6 c.f.u. ml^{-1} of *S. aureus* and incubated at 37 °C for 24 h. To evaluate the effect of the combination, the fractional inhibitory concentration (FIC) was calculated for each drug; then, the FIC index (FICI) was calculated as follows (Oo et al., 2010):

\[ \text{FICI} = \text{MIC of drug in combination/MIC of drug alone} \]

FICI ≤ 0.5, synergy; 0.5 < FICI ≤ 4.0, indifference; FICI > 4.0, antagonism.

**Establishment of microbial biofilms.** The ability of *S. aureus* strains to produce slime was confirmed by culturing the bacteria on Congo red agar as described previously (Freeman et al., 1989). In our study, biofilm cultivation in polystyrene microtitre plates was performed essentially as described by Christensen et al. (1985) and Hübner et al. (2010), with some modifications. Briefly, *S. aureus* was inoculated in 10 ml of TSB-g and incubated for 24 h with shaking at 37 °C. Next, the cultures were diluted 1 : 100, and 200 µl of the diluted cultures per well was inoculated into flat-bottomed 96-well tissue culture polystyrene microtitre plates. After 48 h of incubation at 37 °C, the plates were washed twice with sterile PBS, fixed in Bouin’s fixation (Becton Dickinson) for 1 h and washed again with PBS. Subsequently, the wells were stained with 200 µl of 1% crystal violet for 10 min. Excess crystal violet was removed by gently washing the plate twice with distilled water. Finally, a volume of 250 µl of 95% ethanol solution was added to each well, and the optical density was measured at 570 nm. To compensate for background absorbance, the OD of sterile TSB with fixative and dye was recorded and subtracted from the results. The mean OD 570 nm value was determined using three replicates and was considered to be adherence-positive at an OD 570 nm greater than or equal to 0.12 and adherence-negative at an OD 570 nm less than 0.12.

**Antimicrobial susceptibility testing of *S. aureus** in biofilms.** Cultivated biofilms in flat-bottomed 96-well tissue culture polystyrene microtitre plates were gently washed with 250 µl of PBS to remove non-adherent cells. Serial double dilutions of BBR and totarol were prepared in TSB-g and added to the wells. To triplicate wells, 100 µl TSB-g was added to 100 µl of the antimicrobial agents in rows A–B. Next, the cultures were diluted 1 : 100, and 200 µl of the diluted cultures per well was inoculated into flat-bottomed 96-well tissue culture polystyrene microtitre plates. After 48 h of incubation at 37 °C, the plates were washed twice with sterile PBS, fixed in Bouin’s fixation (Becton Dickinson) for 1 h and washed again with PBS. Subsequently, the wells were stained with 200 µl of 1% crystal violet for 10 min. Excess crystal violet was removed by gently washing the plate twice with distilled water. Finally, a volume of 250 µl of 95% ethanol solution was added to each well, and the optical density was measured at 570 nm. To compensate for background absorbance, the OD of sterile TSB with fixative and dye was recorded and subtracted from the results. The mean OD 570 nm value was determined using three replicates and was considered to be adherence-positive at an OD 570 nm greater than or equal to 0.12 and adherence-negative at an OD 570 nm less than 0.12.
show growth below or equal to that of the control. The minimum biofilm bactericidal concentrations (MBBCs) were identified as the lowest concentration demonstrating no bacterial growth (Hendry et al., 2009; Xing et al., 2012).

**Interactions between BBR and totarol against S. aureus in biofilms.** The flat-bottomed 96-well tissue culture polystyrene microtitre plates seeded with the biofilms were gently washed once with PBS to remove any unbound cells. BBR and totarol were diluted as described previously. A total of 100 µl of BBR was added to the columns with final concentrations ranging from 1/128 to 4-fold the MIC and 100 µl of totarol was added to the rows with final concentrations ranging from 1/16 to 4-fold the MIC. The plates were incubated at 37 °C for 24 h. Following incubation, all BBR and totarol was removed and the wells were washed once with PBS. The MICs were determined as described previously and the FICI was calculated. The biofilm matrix (Wang et al., 2010) was quantified as described previously in a microplate spectrophotometer set at 595 nm (Durham-Colleran, 2007). Briefly, S. aureus 3101 biofilms treated with BBR and totarol alone or in combination were treated with BBR and totarol alone or in combination. The flat-bottomed 96-well tissue culture polystyrene microtitre plates seeded with the biofilms were gently washed carefully with PBS and stained with 200 µl of crystal violet in water for 15 min. Excess stain was gently rinsed off and the wells were washed once with PBS. The MICs were determined as described previously and the FICI was calculated. The assay was performed in duplicate microtitre plates on different days.

**Confocal laser scanning microscopy (CLSM) of biofilms.** Images of biofilms were collected following treatment with BBR and totarol alone or in combination in addition to the growth control. Aliquots (2 ml) collected from diluted TSB-g following a 24 h incubation were used for biofilm growth on coverslips in 6-well dishes for 48 h. Then, the coverslips were washed carefully with PBS, moved to a new plate and treated for 24 h with BBR and totarol alone or in combination. The coverslips were washed again with PBS and stained with a LIVE/DEAD BacLight Bacterial Viability kit (Invitrogen Molecular Probes) following the manufacturer’s instructions. CLSM images were collected using an Olympus FV1000 confocal laser scanning microscope (Olympus) with a 60 × objective lens. Image analyses and export were performed with Fluoview version 1.7.3.0.

**Biofilm adhesion assays.** The selected strain S. aureus 3101 was cultured in 5 ml of TSB-g for 24 h at 37 °C and diluted 1/50 in fresh TSB-g, yielding a final concentration of approximately 1 × 10^6 cfu/200 µl. This suspension was added to each of a 96-well tissue culture microtitre plate and incubated for 48 h at 37 °C. TSB-g was used as the negative control. After incubation, the waste medium was gently aspirated, the wells of the plates were washed with PBS and the biofilms were treated with BBR and totarol alone or in combination. Then, the wells were rinsed with PBS and stained with 200 µl of 0.1 % crystal violet in water for 15 min. Excess stain was gently rinsed off with PBS and the plates were air-dried. The amount of biofilm biomass was quantified by destaining the wells with 200 µl of 33 % acetic acid and then measuring the absorbance of the crystal violet solution in a microplate spectrophotometer set at 595 nm (Durham-Colleran et al., 2010).

**Quantification of biofilm eDNA.** Extracellular DNA (eDNA) is found in S. aureus biofilms and contributes to the strength of the biofilm matrix (Wang et al., 2003); moreover, eDNA plays a biological role in bacterial programmed cell death and cidA-mediated lysis (Rice et al., 2007). The eDNA was extracted from the biofilms and quantified using spectrophotometry. Extracellular genomic DNA isolation from biofilms was performed as described previously (Rice et al., 2007). Briefly, S. aureus 3101 biofilms treated with BBR and totarol alone or in combination for 24 h were chilled at 4 °C for 1 h and then treated with 1.0 µl of 0.5 M EDTA. The supernatants were discarded and the unwashed biofilms were resuspended in 50 mM TES buffer [Tris/HCl (pH 8.0), 10 mM EDTA, and 500 mM NaCl]. eDNA was extracted with phenol/chloroform/isooamyl alcohol (25 : 24 : 1), precipitated with 100 % ethanol and dissolved in 20 µl of TE buffer. The concentration and purity of the purified DNA were determined spectrophotometrically by calculating the A260/A280 absorbance ratio using a NanoDrop 2000 (Thermo Scientific) (Steinberger & Holden, 2005).

**Detection of PIA expression.** The main exopolysaccharide of the S. aureus biofilm matrix is PIA/PNAG (Arciola et al., 2001). Polysaccharide intercellular adhesion (PIA) synthesis is mediated by the intercellular adhesion (ica) operon (Cramton et al., 1999; Heilmann et al., 1996). Here, a dot-blot assay was used to detect the production of PIA in the biofilm extracellular matrix. PIA was detected as described previously (Lou et al., 2011). Briefly, S. aureus strains were grown in 6-well plates under static conditions at 37 °C for 24 h. Then, the cells were scraped off and resuspended in 0.5 M EDTA (pH 8.0). The supernatant was treated with proteinase K (final concentration 4 mg ml⁻¹; Roche) for 3 h at 37 °C. Serial dilutions of the PIA extract were transferred to a nitrocellulose membrane (Millipore). The air-dried membrane was blocked with 3 % (w/v) BSA, and subsequently incubated with 3.2 µg ml⁻¹ wheatgerm agglutinin coupled to horse-radish peroxidase (WGA–HRP conjugate; Sigma) for 1 h. The HRP activity was visualized via chromogenic detection.

**RNA isolation and real-time reverse transcriptase (RT)-PCR.** The expression of biofilm-related genes (cidA, icaA and sarA) was analysed by real-time RT-PCR. Bacterial cells were suspended in 0.5 mg of lysostaphin (Sigma-Aldrich) ml⁻¹ and incubated at 37 °C for 15 min. Then, RNA was isolated and purified using the RNAeasy Mini kit (Qiagen) according to the manufacturer’s instructions. Quantitative real-time PCR was performed in triplicate using the 7000 Sequence Detection System (Applied Biosystems) according to a previously described procedure (Wang et al., 2008). The cDNA was subjected to real-time PCR using the primer pairs listed in Table 1.

**Statistical analysis.** Three independent trials were conducted for each treatment. Statistical analyses were conducted using SPSS 16 software. Statistical analysis was performed using ANOVA performed on the indicated datasets. ANOVA followed by Tukey’s post-hoc analysis was used to compare the differences between groups. Pairwise comparisons with differences of P<0.05 were considered statistically significant; P<0.01 was considered extremely significant.

**Table 1.** Primers used in this study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Primer sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sarA</td>
<td>sarA-F</td>
<td>TCTTGTTTAAATGCGACAAACAGTAA</td>
<td>Abdelhady et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>sarA-R</td>
<td>TGGTTTCTCAGTATTGGTTT</td>
<td></td>
</tr>
<tr>
<td>icaA</td>
<td>icaA-F</td>
<td>GTCAAGACACTTGCTGGCCCA</td>
<td>Hsu et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>icaA-R</td>
<td>GAGCCCATCTCACGGTTGC</td>
<td></td>
</tr>
<tr>
<td>cidA</td>
<td>cidA-F</td>
<td>AGGGTAATTTTGAGACAAATCCA</td>
<td>Hsu et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>cidA-R</td>
<td>CCCCTAGCCCGACTATTGTTGTC</td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>16S rRNA-F</td>
<td>GCGTGCCCTTGTATTATC</td>
<td>Qiu et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>16S rRNA-R</td>
<td>AGATGGTGGTFAAGTCC</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

Determination of drug MICs and MBCs in suspension and biofilm cultures

The MICs and MBCs of BBR alone against all tested strains in suspension cultures ranged from 32 to 64 \( \mu \text{g ml}^{-1} \) and from 512 to >1024 \( \mu \text{g ml}^{-1} \), respectively; the MIC and MBC values for totarol were 0.5 to 2 \( \mu \text{g ml}^{-1} \) and 4 to 8 \( \mu \text{g ml}^{-1} \), respectively (Table 2).

Using the microtitre plate assay, all ten isolates of MRSA and ATCC 29213 that had an OD greater than 0.12 were determined to be biofilm-positive strains. Next, we investigated the susceptibility of \( S. aureus \) biofilms to BBR and totarol. All strains grown in biofilms were sensitive to both drugs, with the MBICs ranging from 128 to 256 \( \mu \text{g ml}^{-1} \) for BBR and 4 to 8 \( \mu \text{g ml}^{-1} \) for totarol. This result suggests that totarol is an effective antimicrobial agent that can inhibit \( S. aureus \) biofilm formation. The finding that BBR had higher biofilm MBBC values (1024 to \( 1024 \mu \text{g ml}^{-1} \)) compared to totarol (32 to 512 \( \mu \text{g ml}^{-1} \)) (Table 3) indicated that BBR possessed certain antibacterial effects. Additionally, this finding demonstrates that totarol had better antibacterial activity against planktonic \( S. aureus \) than biofilm \( S. aureus \).

Drug susceptibility in combination against \( S. aureus \) in suspension and biofilm cultures

The interaction between BBR and totarol was synergistic in all 11 strains of \( S. aureus \) grown in biofilms according to the FICI method (all FICI values ranged from 0.188 to 0.5). The results showed that no antagonistic activity was observed for BBR and totarol for any of the strains grown in suspension, with the FICI values in the range 0.313–1.00 (Table 4). Thus, the synergism between BBR and totarol against \( S. aureus \) in biofilms was much stronger than the interaction in suspension cultures.

\( S. aureus \) 3101 was tested in all of the following sections, because the FICI values in the biofilms of this strain were lower and steadier compared to the other strains and the FICI values in the suspension culture were the lowest among the \( S. aureus \) strains.

Adhesion assays and CLSM of bacterial cell survival in biofilms exposed to BBR or totarol alone or in combination

Treatment with BBR and totarol alone for 24 h at the MBICs inhibited the biofilm formation of \( S. aureus \) strain 3101 by 45.5 % and 37.0 %, respectively, compared to the control (Fig. 1). However, the inhibition ratio of the biofilms reached 84.5 % following combination treatment with BBR and totarol. Compared to BBR and totarol alone, the combination of the two drugs was considered to be extremely significant (\( P<0.01 \)). CLSM images are shown in Fig. 2. A confluent mixture and an abundance of cell clusters were observed in the control group (Fig. 2a). Treatment of a 48 h biofilm with BBR at the MBIC value (128 \( \mu \text{g ml}^{-1} \)) decreased the number of bacteria in the biofilm by a small amount. Additionally, the interstice between the bacterial cells was slightly widened (Fig. 2b). Exposure to totarol at the MBIC concentration (4 \( \mu \text{g ml}^{-1} \)) (Fig. 2c) resulted in dispersion and lysis of the bacterial cells compared to the control group. Both drugs reduced the biofilm thickness, although the number of viable cells was still high. The combination of the two drugs (128 \( \mu \text{g ml}^{-1} \) BBR plus 4 \( \mu \text{g ml}^{-1} \) totarol) resulted in biofilm disintegration and nearly complete cell death (Fig. 2d).

Table 2. Susceptibility of BBR or totarol alone or in combination against the \( S. aureus \) strains in suspension

<table>
<thead>
<tr>
<th>Strains</th>
<th>Median MIC and MBC (range) of drug (( \mu \text{g ml}^{-1} ))</th>
<th>BBR Alone</th>
<th>Totarol Alone</th>
<th>In combination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td>( S. aureus ) 3629</td>
<td>32 (32)</td>
<td>512 (256–512)</td>
<td>1 (1)</td>
<td>8 (4–8)</td>
</tr>
<tr>
<td>( S. aureus ) 2985</td>
<td>64 (64–128)</td>
<td>512 (512)</td>
<td>0.5 (0.5–1)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>( S. aureus ) 3800</td>
<td>32 (32–64)</td>
<td>256 (256–512)</td>
<td>1 (0.5–1)</td>
<td>4 (4–8)</td>
</tr>
<tr>
<td>( S. aureus ) 1862</td>
<td>64 (64)</td>
<td>512 (512)</td>
<td>2 (1–2)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>( S. aureus ) 3101</td>
<td>64 (64–128)</td>
<td>&gt;512</td>
<td>0.5 (0.5)</td>
<td>4 (2–4)</td>
</tr>
<tr>
<td>( S. aureus ) 3303</td>
<td>64 (64)</td>
<td>512 (512)</td>
<td>1 (1)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>( S. aureus ) 3015</td>
<td>32 (32)</td>
<td>512 (256–512)</td>
<td>1 (1)</td>
<td>4 (4–8)</td>
</tr>
<tr>
<td>( S. aureus ) 3218</td>
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<td>&gt;512</td>
<td>0.5 (0.5)</td>
<td>4 (2–4)</td>
</tr>
<tr>
<td>( S. aureus ) 3701</td>
<td>64 (64)</td>
<td>512 (512)</td>
<td>0.5 (0.5–1)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>( S. aureus ) 29213</td>
<td>32 (32)</td>
<td>512 (512)</td>
<td>2 (2)</td>
<td>8 (8–16)</td>
</tr>
</tbody>
</table>

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Effect of BBR or totarol alone or in combination on eDNA release from *S. aureus*

Previous studies demonstrated that eDNA is a critical component for bacterial adhesion during the initial stage of biofilm development (Das et al., 2010; Whitchurch et al., 2002). The amounts of eDNA present in the cell-free supernatants from the biofilms treated with BBR or totarol alone or in combination are shown in Fig. 3. The average amount of eDNA present in bacteria treated with subinhibitory concentrations of BBR (16 µg ml\(^{-1}\)) or totarol (0.5 µg ml\(^{-1}\)) alone was 4.3-fold and 1.6-fold less than the amount present in the untreated group, respectively. Furthermore, the amount of eDNA in the combination-treated biofilm was reduced 32-fold compared with the control biofilm. The results revealed that there was an extremely significant difference (\(P < 0.01\)) in the amount of eDNA present between the biofilms treated with BBR and totarol in combination compared to BBR or totarol treatment alone.

Table 4. *In vitro* interaction between BBR and totarol against against the *S. aureus* strains in suspension and in biofilm

For the FICI model, synergy was defined as an FICI of \(\leq 0.5\), antagonism as an FICI of \(>4.0\), and indifference (i.e. no interaction) as an FICI of \(>0.5\) to 4.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Median MBIC and MBBC (range) of drug (µg ml(^{-1}))</th>
<th>Alone</th>
<th>In combination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BBR</td>
<td>MBIC</td>
<td>MBBC</td>
</tr>
<tr>
<td>S. aureus 3629</td>
<td>128 (64–128)</td>
<td>&gt;1024</td>
<td>4 (4)</td>
</tr>
<tr>
<td>S. aureus 2985</td>
<td>128 (128)</td>
<td>&gt;1024</td>
<td>4 (4–8)</td>
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<tr>
<td>S. aureus 3800</td>
<td>256 (128–256)</td>
<td>&gt;1024</td>
<td>8 (4–8)</td>
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<tr>
<td>S. aureus 1862</td>
<td>128 (128)</td>
<td>1024 (1024)</td>
<td>4 (4)</td>
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<tr>
<td>S. aureus 3101</td>
<td>256 (256)</td>
<td>&gt;1024</td>
<td>8 (8)</td>
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<tr>
<td>S. aureus 3303</td>
<td>256 (128–256)</td>
<td>&gt;1024</td>
<td>4 (2–4)</td>
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<tr>
<td>S. aureus 3015</td>
<td>128 (128)</td>
<td>1024 (1024)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>S. aureus 3218</td>
<td>256 (128–256)</td>
<td>&gt;1024</td>
<td>4 (4–8)</td>
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<tr>
<td>S. aureus 1987</td>
<td>256 (256)</td>
<td>&gt;1024</td>
<td>8 (4–8)</td>
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<td>S. aureus 3701</td>
<td>128 (128)</td>
<td>&gt;1024</td>
<td>8 (8)</td>
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<tr>
<td>S. aureus 29213</td>
<td>128 (128)</td>
<td>&gt;1024</td>
<td>4 (4)</td>
</tr>
</tbody>
</table>

ANT, Antagonism; IND, indifference; SYN, synergism.
PIA production levels in biofilms treated with BBR and totarol in combination were significantly reduced (P<0.01) in comparison with biofilms treated with BBR or totarol alone (Fig. 4). The inhibition ratio of PIA expression following exposure to subinhibitory concentrations of BBR (16 µg ml⁻¹) or totarol (0.5 µg ml⁻¹) was 17.3 % or 10.9 %, respectively, but the inhibition ratio reached 82.3 % when exposed to BBR and totarol in combination (Fig. 4). Thus, the observed reduction in PIA production may be one of the primary factors responsible for the altered phenotype of decreased biofilm formation.

Gene activities modulated by exposure to subinhibitory concentrations of BBR or totarol alone or in combination

To determine whether the influence manifested at the transcriptional level, RNA was isolated from S. aureus strain 3101 following treatment with subinhibitory concentrations of BBR (16 µg ml⁻¹) and totarol (0.5 µg ml⁻¹) alone or in combination for 24 h. Real-time RT-PCR results showed that the transcript levels of cida in biofilms treated with BBR and totarol were decreased by 3-fold and 1.7-fold, respectively; the level observably decreased following combination treatment with the two drugs. The gene icaA, which is essential for PIA synthesis, was inhibited by more than 4.7-fold following treatment with BBR and totarol in combination (Fig. 5). Similar to the results shown in Fig. 5, the combination treatment group exhibited a significant decrease in the transcript levels of sarA compared to treatment with BBR or totarol alone. The decrease in the transcript levels of the three biofilm-related genes in the combination group compared to BBR or totarol treatment alone was significant (P<0.01).

**DISCUSSION**

The requirement for new antibiotics to overcome bacterial resistance in suspension and biofilm cultures has led to the investigation of novel drug therapies. In this study, we used the chequerboard dilution method to analyse the antimicrobial activity of totarol alone or in combination with BBR against ten clinical MRSA isolates and the QC strain ATCC 29213 grown in suspension and biofilm cultures. The current results demonstrated that both drugs had efficient antibiotic activities. The interaction between totarol and BBR against the 11 S. aureus strains in suspension was ranked as synergy or indifference. However, the combination of the two drugs showed synergistic activity against all tested strains of S. aureus grown in biofilms; the results were verified by CLSM and adhesion assays. The different interactions of totarol and BBR against S. aureus in suspension and biofilms revealed that the combination of totarol and BBR would be clinically effective alternatives for the treatment of patients with biofilm-associated MRSA infections.

In the present study, BBR in conjunction with totarol significantly reduced the amount of eDNA and PIA production present in the biofilm compared to treatment with BBR or totarol alone (P<0.01). Additionally, the expression of the murein hydrolase inhibitor cida was investigated by real-time RT-PCR. Recent studies have demonstrated that cida has a positive effect on murein hydrolase activity. Furthermore, a cida mutant biofilm displayed decreased cell lysis and release of eDNA (Rice et al., 2007). Our results revealed that the expression levels of cida following treatment with BBR or totarol alone or in combination were in agreement with the changes in eDNA levels. Quantitative RT-PCR revealed that combination treatment inhibited the amount of icaA mRNA expressed by the cells more than 4.7-fold compared with the control (Fig. 5). The enzymes required for PIA synthesis are encoded by the ica operon; mutations in this operon resulted in a reduced capacity to form a biofilm in both S. aureus and S. epidermidis (Cramton et al., 1999; Heilmann et al., 1996). Hence, we can conclude that BBR or totarol alone or in combination significantly inhibited (P<0.01) biofilm formation by repressing the transcriptional level of the ica operon and thereby reducing PIA synthesis. sarA has a global effect on many S. aureus virulence genes that seem to play a role in biofilm formation [e.g. multiple extracellular proteases, nuclease, and fibronectin-binding proteins (FnBPs)] (Dunman et al., 2001). Thus, we tested sarA gene expression in our...
study and demonstrated that the combination treatment induced significantly decreased ($P<0.01$) sarA gene expression in $S. \text{aureus}$ strain 3101 in vitro compared to treatment with BBR or totarol alone.

Previous studies showed that totarol possessed antibacterial activity, especially against MRSA (Kubo et al., 1992). However, the mechanism underlying its antimicrobial action is not known. Efflux is a universal resistance mechanism among bacteria. EPIs, which are simple, effective and cheap chemicals, can decrease efflux activity and change the pump components (Pagès et al., 2005). Moreover, EPIs have been considered to be effective agents against drug-resistant strains because they are well tolerated in humans (Marquez, 2005). One report showed that totarol was an active EPI and could reduce NorA-mediated EtBr efflux in $S. \text{aureus}$ (Smith et al., 2007); moreover, totarol could also inhibit bacterial respiratory transport (Haraguchi et al., 2008).

**Fig. 2.** CLSM image of LIVE/DEAD-stained $S. \text{aureus}$ 3101 biofilms grown on coverslip discs. (a) Growth control; (b) $S. \text{aureus}$ 3101 treated with 128 $\mu$g ml$^{-1}$ BBR; (c) $S. \text{aureus}$ 3101 treated with 4 $\mu$g ml$^{-1}$ totarol and (d) $S. \text{aureus}$ 3101 treated with 4 $\mu$g ml$^{-1}$ totarol in the presence of 128 $\mu$g ml$^{-1}$ BBR.

**Fig. 3.** Effect of eDNA release on $S. \text{aureus}$. The amount of eDNA in the cell-free supernatants from $S. \text{aureus}$ 3101 biofilms treated with BBR (16 $\mu$g ml$^{-1}$) or totarol (0.5 $\mu$g ml$^{-1}$) alone or in combination was measured by spectrophotometry. The values are expressed as nanograms of eDNA per relative biofilm biomass (OD 600). Values represent the mean±SD for three independent experiments. * $P<0.05$; ** $P<0.01$. 
et al., 1996). These findings may represent the mechanisms underlying its antibacterial activity against MRSA strains.

In the current study, we assayed totarol against a clinical isolate and found significant antimicrobial activity against MRSA strains grown in biofilms. BBR is an active antimicrobial agent towards *S. aureus* grown in suspension cultures that has the ability to intercalate DNA and inhibit DNA or protein synthesis in micro-organism cells (Cernáková & Kostálová, 2002). However, few reports have explored its effect on *S. aureus* biofilm cultures or the mechanism underlying its activity. Our study investigated the antibiofilm activity of BBR against *S. aureus* and showed that it had a weak antibiofilm effect.

BBR has been reported to be the substrate of the chromosomally encoded *S. aureus* multi-drug resistance (MDR) pump NorA. Indeed, the MDR-dependent efflux of BBR from *S. aureus* cells was severe (Lewis, 2001; Stermitz et al., 2000). This finding may explain why BBR exhibits a relatively weak antibacterial effect against biofilms with higher MICs. Additionally, both application of an MDR inhibitor and disruption of the main MDR (NorA) can strongly strengthen the antibiofilm of BBR (Hsieh et al., 1998). As mentioned above, totarol is an active EPI that could reduce NorA-mediated EtBr efflux in *S. aureus* (Smith et al., 2007). Thus, the administration of BBR in combination with totarol could substantially increase BBR’s antimicrobial action. This hypothesis was in line with our results, because the MBIC of BBR in combination against biofilms was reduced 4- to 16-fold lower compared to its use alone.

Micro-organisms may reside on surfaces in aggregates embedded in a biofilm, rendering them less susceptible to cleaning and disinfection. Furthermore, many medical devices such as central venous catheter hubs and needleless connectors also become colonized with micro-organisms capable of producing a biofilm (Casey et al., 2003). The synergistic activity between BBR and totarol may represent
clinically effective alternatives for treating patients with biofilm-associated MRSA infections, such as catheter-related or prosthetic joint infections. While much of the research data advocates the potential use of BBR and totarol in a clinical setting to prevent and treat infections (Sarkar et al., 2011; Kim & Shaw, 2010), there is little information regarding their actual application in the clinic, which needs to be taken into consideration. Therefore, animal experiments and clinical studies are required to validate BBR and totarol combination regimens for the clinical management of MRSA biofilm and mixed-species biofilm infections.

ACKNOWLEDGEMENTS

Financial support for this work came from the following sources: the National Nature Science Foundation of China (No. 31271951, No. 31000822 and No. 31172364), the Program for New Century Excellent Talents in University (NCET-13-0245), China Postdoctoral Science Foundation (2013M530142), National Key Basic Research Program (973 Program) (No. 2013CB127205) and the Important National Science and Technology Specific Projects (2012ZX10003002).

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