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Synthesis of ruthenium complexes functionalized with benzothiophene and their antibacterial activity against *Staphylococcus aureus*†

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New effective antimicrobial agents with novel modes of action are urgently needed due to the continued emergence of drug-resistant bacteria. Here, three ruthenium complexes functionalized with benzothiophene: $[Ru(phen)_2(BTPIP)](ClO_4)_2$ (**Ru(II)-1**), $[Ru(dmp)_2(BTPIP)](ClO_4)_2$ (**Ru(II)-2**) and $[Ru(dmb)_2(BTPIP)](ClO_4)_2$ (**Ru(II)-3**) (dmb = 4,4′-dimethyl-2,2′-bipyridine, phen = 1,10-phenanthroline, dmp = 2,9-dimethyl-1,10-phenanthroline) have been synthesized and their antimicrobial activities *in vitro* were assessed. Minimum inhibitory concentration (MIC) assays indicated that the three **Ru(II)-1**, **Ru(II)-2** and **Ru(II)-3** complexes all showed antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The most active **Ru(II)-3** complex was further tested against biofilms. Furthermore, it was also tested whether complex **Ru(II)-3** could serve as an antibacterial adjuvant. Interestingly, the checkerboard data showed that **Ru(II)-3** selectively exhibited synergism with aminoglycoside antibiotics. More importantly, the observed synergetic effect might be attributed to the inhibition of the regulatory function of *Sa*CcpA. Finally, *in vivo* bacterial infection treatment studies through a murine skin infection model and skin irritation test were also conducted. All in all, these results confirmed that ruthenium complexes functionalized with benzothiophene have good antimicrobial activity against *Staphylococcus aureus*.

Introduction

Today, antibiotics have become the cornerstone of modern medicine.[1] However, the continued emergence of drug-resistant bacteria, coupled with the decline in the discovery of new antimicrobial drugs in the pharmaceutical pipeline, has created a public health crisis.[2–4] *Staphylococcus aureus* (*S. aureus*) is the leading cause of both hospital and community associated infections worldwide and a principal cause of morbidity and mortality. It is worth noting that *S. aureus* is a commensal that colonizes the nares, axillae, vagina, pharynx, or damaged skin surfaces.[5] This pathogen can infect almost every tissue and produces various virulence factors in a human host.[6] Worse still, resistance to vancomycin, linezolid and daptomycin has already been reported in clinical MRSA (methicillin-resistant *S. aureus*) strains.[7–9] Obviously, there is clearly a need for the development of new antimicrobials. More importantly, there is a pressing need for new effective antimicrobial agents with novel modes of action.[10]

Compared to traditional organic antimicrobial drugs, metal complexes are promising in this regard as they offer alternative electronic and stereochemical properties. It is worth noting that the antimicrobial activity of transition metal complexes has been widely tested in recent years.[11–16] For example, silver or copper(II)-based complexes were reported to show meaningful antimicrobial activity.[16–18] Interestingly, polypyridyl ruthenium(II) complexes were reported to show significant bactericidal activity against methicillin-resistant *S. aureus* strains.[19] More importantly, ruthenium coordinated to established organic drugs could enhance their antimicrobial activities.[20] In a previous study, we designed and synthesized a series of ruthenium polypyridyl complexes. The antibacterial activity studies indicated that the ruthenium complexes formed by the benzothiophene-substituted ligands showed better antibacterial activity.[21]

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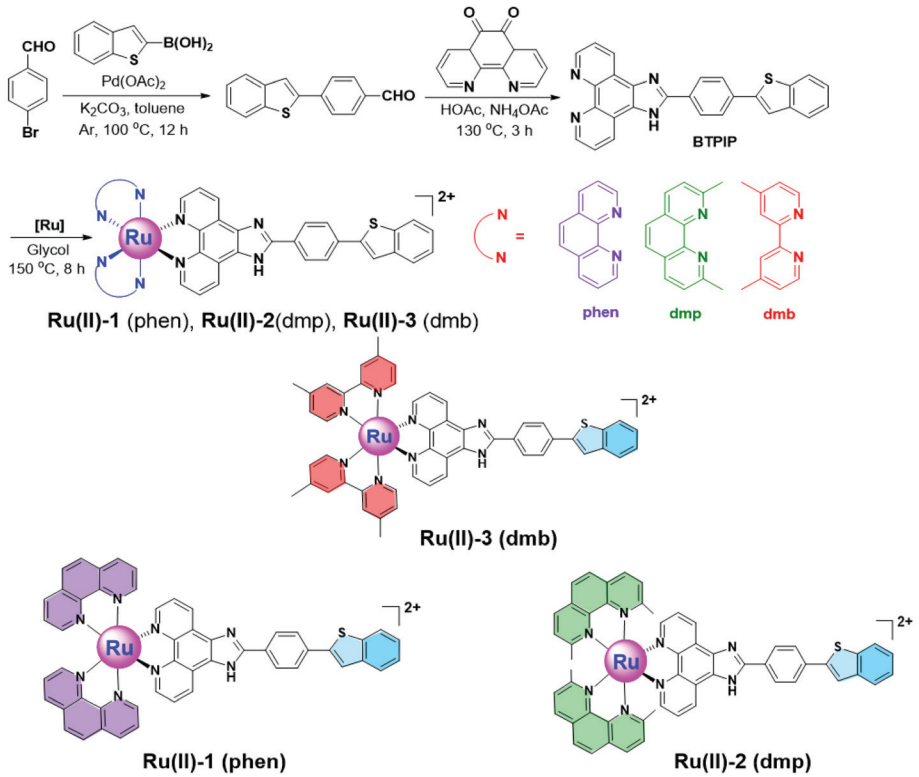


Scheme 1 The synthetic route of ligand and ruthenium complexes.

As ruthenium complexes formed by the benzothiophene-substituted ligands have strong antibacterial potential, we aimed to develop novel ruthenium complexes with better activity based on a benzothiophene-substituted ligand. For this purpose, we designed and synthesized three ruthenium complexes functionalized with benzothiophene: [Ru(phen)$_2$(BTPIP)] (ClO_4)$_2$ (**Ru(II)-1**), [Ru(dmp)$_2$(BTPIP)] (ClO_4)$_2$ (**Ru(II)-2**) and [Ru(dmb)$_2$(BTPIP)](ClO_4)$_2$ (**Ru(II)-3**) (Scheme 1). Their antimicrobial activities against *S. aureus* and *P. aeruginosa* were investigated. Subsequently, the **Ru(II)-3** complex which exhibited the best bactericidal effect was further tested against biofilms. In addition, in order to investigate whether ruthenium complexes could also serve as antibiotic adjuvants, studies of the synergism between **Ru(II)-3** and common antibiotics against *S. aureus* were performed. Then the possible mechanism of the observed synergetic effect was investigated. Finally, the antimicrobial activities of **Ru(II)-3** *in vivo* were investigated through a murine skin infection model and a skin irritation test were also conducted to investigate the irritation effect of **Ru(II)-3** on the skin.

Experimental

Chemistry

Synthesis of ligand (BTPIP). A mixture of 1,10-phenanthroline-5,6-dione (100.00 mg, 0.5 mmol), 4-(benzo[*b*]thiophen-2-yl)benzaldehyde (119.0 mg, 0.5 mmol), ammonium acetate (15 mmol, 1156.2 mg) and acetic acid (30 mL) was refluxed with stirring for 4 h. The cooled solution was diluted with water and neutralized with concentrated aqueous ammonia. The brown precipitate was collected and purified by column chromatography on silica gel (60–100 mesh) with ethanol as eluent to give the compound as a brown yellow powder. Yield: 188.3 mg, 88%. IR: ν = 3101, 2102, 1916, 1793, 1691, 1597, 1588, 1480, 1358, 1312, 1188, 1121, 953, 819, 736, 682 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.03 (d, J = 4.0 Hz, 2H), 8.94 (d, J = 8.1 Hz, 2H), 8.40 (dd, J = 22.3, 8.3 Hz, 2H), 7.99 (d, J = 10.1 Hz, 5H), 7.88–7.80 (m, 3H), 7.40 (t, J = 6.8 Hz, 2H), 3.49 (s, 0H); HRMS (ESI) m/z: calcd for $C_{27}H_{17}N_4S$ [M + H]$^+$, 429.1168; found 429.1169.

Synthesis of [Ru(phen)$_2$(BTPIP)](ClO_4)$_2$ (Ru(II)-1). A mixture of *cis*-[Ru(phen)$_2$Cl$_2$]·2H$_2$O (170.4 mg, 0.3 mmol) and BTPIP (128.4 mg, 0.3 mmol) in ethylene glycol (12 mL) was heated at 150 °C under argon for 8 h to give a clear red solution. Upon cooling, a red precipitate was obtained by dropwise addition of saturated aqueous $NaClO_4$ solution. The crude product was purified by column chromatography on neutral alumina with a mixture of CH_3CN–toluene (1 : 1, v/v) as eluent. The red band was collected. The solvent was removed under reduced pressure and a red powder was obtained. Yield: 254.8 mg, 78%. IR: ν = 3051, 1601, 1576, 1508, 1478, 1455, 1426, 1363, 1304, 1250, 1224, 944, 843, 808, 750, 623, 528 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 9.13 (dd, J = 24.7, 7.9 Hz, 1H), 8.90 (dd, J = 14.6, 8.1 Hz, 2H), 8.47 (dd, J = 41.7, 7.2 Hz, 1H), 8.24 (t, J = 7.8 Hz, 2H), 8.13 (t, J = 7.7 Hz, 2H), 8.00 (t, J = 9.7 Hz, 4H),

7.96–7.81 (m, 7H), 7.70–7.61 (m, 4H), 7.41 (d, J = 6.3 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 157.3, 157.1, 151.9, 149.1, 149.2, 149.3, 145.2, 145.1, 143.1, 141.0, 139.2, 138.4, 138.2, 134.5, 130.9, 128.4, 128.2, 127.6, 127.7, 126.8, 126.2, 126.3, 125.4, 125.5, 124.9, 125.0, 124.8, 124.4, 123.0, 121.0, 115.4; HRMS (ESI) m/z: calcd for $C_{51}H_{31}N_8SRu$ $[M - 2ClO_4 - H]^+$, 889.1443; found 889.1451.

Synthesis of $[Ru(dmp)_2(BTPIP)](ClO_4)_2$ (Ru(II)-2). This complex was synthesized in an identical manner to that described for complex $[Ru(phen)_2(BTPIP)](ClO_4)_2$, with *cis*-$[Ru(dmp)_2Cl_2]\cdot 2H_2O$ in place of *cis*-$[Ru(phen)_2Cl_2]\cdot 2H_2O$. Yield: 237.1 mg, 69%. IR: ν = 3053, 1964, 1605, 1588, 1507, 1478, 1433, 1348, 1248, 1216, 1089, 854, 809, 726, 622, 555 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (d, J = 8.3 Hz, 2H), 8.83 (d, J = 8.0 Hz, 2H), 8.48–8.43 (m, 4H), 8.28 (t, J = 10.2 Hz, 4H), 8.02–7.96 (m, 4H), 7.94–7.87 (m, 3H), 7.52–7.44 (m, 2H), 7.43–7.34 (m, 6H), 7.19 (m, J = 34.5, 7.6 Hz, 1H), 1.97 (s, 6H), 1.76 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.4, 166.8, 150.7, 150.3, 149.4, 149.0, 148.3, 145.9, 143.6, 142.9, 140.9, 139.2, 138.6, 137.8, 137.2, 135.0, 131.0, 130.8, 120.0, 129.4, 128.7, 128.0, 127.9, 127.6, 127.0, 126.3, 125.8, 125.4, 124.4, 123.0, 121.3; HRMS (ESI) m/z: calcd for $C_{55}H_{39}N_8SRu$ $[M - 2ClO_4 - H]^+$, 945.2070; found 945.2088.

Synthesis of $[Ru(dmb)_2(BTPIP)](ClO_4)_2$ (Ru(II)-3). This complex was synthesized in an identical manner to that described for complex $[Ru(phen)_2(BTPIP)](ClO_4)_2$, with *cis*-$[Ru(dmb)_2Cl_2]\cdot 2H_2O$ in place of *cis*-$[Ru(phen)_2Cl_2]\cdot 2H_2O$. Yield: 266.6 mg, 81%. IR: ν = 3057, 2919, 1618, 1507, 1479, 1448, 1364, 1304, 1197, 1093, 953, 825, 725, 566, 544 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.06 (dd, J = 13.5, 8.2 Hz, 2H), 8.75 (d, J = 15.5 Hz, 4H), 8.39 (dd, J = 22.9, 7.7 Hz, 2H), 7.98 (m, J = 31.6, 22.2, 5.8 Hz, 10H), 7.70 (s, 2H), 7.48–7.36 (m, 6H), 7.20 (s, 2H), 2.58 (s, 6H), 2.48 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.8, 156.7, 153.6, 151.0, 150.9, 150.0, 149.9, 149.8, 145.6, 142.9, 140.9, 139.3, 135.1, 135.0, 130.7, 130.5, 129.4, 129.0, 128.8, 127.7, 127.0, 127.0, 126.5, 125.5, 125.4, 124.4, 122.96, 121.3, 121.0, 21.2, 21.1; HRMS (ESI) m/z: calcd for $C_{51}H_{39}N_8SRu$ $[M - 2ClO_4 - H]^+$, 897.2069; found 897.2077.

Biology

Bactericidal activity. The bacterial strains were purchased from China Center of Industrial Culture Collection (CICC). The frozen bacteria at −80 °C were scratched on a TSB Agar plate. After growing at 37 °C overnight, a single colony was picked from the TSB Agar plate and cultured in TSB medium. Minimal inhibitory concentrations (MICs) of ruthenium complexes against *S. aureus* (ATCC 25904) and *P. aeruginosa* (ATCC 9027) were measured. In brief, the overnight cultured bacteria were diluted into fresh TSB medium. After culturing for another 2 hours, the bacteria were 1 : 100 diluted with fresh medium to get a bacterial suspension. The MIC assays were performed with a 96-well plate. After the addition of 200 μL of bacterial suspension with different concentrations of ruthenium complexes, the plate was incubated at 37 °C. The antimicrobial activity of the solvent with the same amount of DMSO was tested in the same way and the solvent showed no antimicrobial activity.

Ruthenium complexes inhibited the growth of *S. aureus*. Overnight cultured *S. aureus* strains were 1 : 100 diluted with fresh medium and then further cultured at 37 °C. Subsequently, the logarithmic phase bacteria were 1 : 10 diluted again. After the addition of different concentrations of ruthenium complexes, the bacteria were cultured in a shaker at 37 °C. Then the OD_{600} of the bacteria were measured every 30 minutes.

Thiol detection in *S. aureus*. At first, different concentrations of **Ru(II)-3** were added into the logarithmic phase bacteria. After further culturing for 30 min, the bacteria were collected by centrifugation and washed twice with PBS. Finally, a Thiol Detection Assay Kit (Cayman) was used to determine the concentration of the free thiol in *S. aureus*.

The effect of Ru(II)-3 on biofilm formation. The previous day, 1 mL of 10% albumin from bovine serum were added into 24-well microtiter plates and stored at 4 °C. The albumin from bovine serum was removed from the plate before use. Overnight cultured *S. aureus* strains were 1 : 100 diluted into fresh TSB medium. After further culturing for 5 h, the bacteria were diluted 1 : 200 into TSB medium containing 0.5% glucose and different concentrations of **Ru(II)-3**. Subsequently, 1 mL aliquots of the bacterial suspension were added into the 24-well plate. After culturing at 37 °C for 36 h, the bacterial suspension was removed and the plate was washed with PBS three times. The adherent bacteria were dried out overnight at room temperature and were then strained through 0.1% crystal violet solution (Sangon, China). The crystal violet solution was removed and the plate washed with PBS again after 15 minutes. The biofilm formation could be determined through monitoring the absorbance at 595 nm after the addition of 1 mL of acetic acid.

The synergism study between Ru(II)-3 and antibiotics. At first, the MIC values of all selected antibiotics against *S. aureus* Newman were examined, as described previously.[22] Next, overnight cultured *S. aureus* were 1 : 100 diluted into fresh TSB medium and further incubated until the OD_{600} reached 1. The bacteria were 1 : 20 diluted into fresh TSB medium to obtain a bacterial suspension. Subsequently, 200 μL of bacterial suspension with a gradient concentration of **Ru(II)-3** and antibiotics were transferred into the wells of 96-well plates. After incubation for 20 h at 37 °C, the fractional inhibitory concentration index (FICI) was calculated. GraphPad Prism software was employed for plotting isobolograms.

Real-time PCR investigation of the transcription level of gene. *S. aureus* Newman were cultured in TSB medium the night before. Then the overnight bacteria were 1 : 100 diluted into fresh TSB and cultured at 37 °C. After the bacteria had grown into a logarithmic phase, different concentrations of **Ru(II)-3** were added. *S. aureus* was collected by centrifugation after another 1 hour's incubation at 37 °C. The SV total RNA isolation system (Promega) was used for total RNA extraction. Subsequently, a GoTaq qPCR Master Mix kit (Promega) was employed to investigate the transcription level of the gene

through real-time PCR (Life Technologies). Relative transcription levels were calculated using the $^{-\Delta\Delta}C_t$ method by setting 16S rRNA as an internal control. The values of the untreated group were set as 1. The **Ru(II)-3** treated groups were normalized to that of control groups. The primers used were: 16S rRNA-For and 16S rRNA-Rev, *pckA*-For and *pckA*-Rev, *ccpA*-For and *ccpA*-Rev, *citZ*-For and *citZ*-Rev.

Investigation of the antibacterial activity *in vivo*. First, hair removal cream (Veet®) was smeared on the infection site of female BALB/c mice (6–8 weeks of age, 20–25 g in weight) to remove the hair one day before infection. Subsequently, overnight cultured *S. aureus* Newman strains were 1 : 100 diluted in fresh TSB medium and grown at 37 °C until OD_{600} reached 0.6. The bacteria were harvested by centrifugation and the cell pellet was washed with PBS three times for further use. The abscesses on the mice were developed through subcutaneous injection of 50 μL of *S. aureus* Newman (3×10^7 CFU) which was resuspended with PBS. All the mice were divided into three groups (n = 5 for each group) including a control and a treatment group (**Ru(II)-3**, 0.05 mg mL^{-1} or 0.10 mg mL^{-1}). Before treatment, sterile cream was prepared by mixing stearic acid (4.8 g), glycerin monostearate (1.4 g), liquid paraffin (2.4 g), albolene (0.4 g), lanum (2.0 g), and distilled water (29 g). Subsequently, the mice received twice-daily treatment through cream containing different concentrations of **Ru(II)-3** smeared onto the abscesses. After 64 hours, all the mice were sacrificed and the abscess tissues were collected. Tissue homogenates were serially diluted in PBS and then spread onto agar plates for the enumeration of bacterial load.

Acute dermal irritation test. Female BALB/c mice were randomly divided into three groups: Control, **Ru(II)-3** (0.05 mg mL^{-1}) and **Ru(II)-3** (0.10 mg mL^{-1}). The hair coat of the dorsal area from female mice was clipped and removed on the day before the test. Compounds and a distilled water control were softly attached to the shaved part (about 2 cm^2) once a day for three days. On the fourth day the mice were killed by cervical dislocation and their shaved parts were removed and fixed in 4% paraformaldehyde at 4 °C for 1 day, then embedded in paraffin. Sequential sections were prepared for HE assays.

Results and discussion

The antibacterial activity studies

First of all, we evaluated the antibacterial activity of three ruthenium complexes functionalized with benzothiophene against *S. aureus* (Newman) using minimum inhibitory concentration (MIC) assays. From the results in Table 1, as we expected, the three compounds all showed good antibacterial activity against *S. aureus*, with MIC ranging from 0.003 to 0.050 mg mL^{-1}. Interestingly, $[Ru(dmb)_2(BTPIP)](ClO_4)_2$ (**Ru(II)-3**) showed the best antibacterial effect against *S. aureus*. Compared with ruthenium complexes formed by 2,2′-bipyridine which we previously reported ($[Ru(bpy)_2(BTPIP)](ClO_4)_2$, MIC = 0.016 mg mL^{-1}), $[Ru(phen)_2(BTPIP)](ClO_4)_2$ (**Ru(II)-1**) formed with 1,10-phenanthroline showed weaker antibacterial activity. This result indicated that 2,2′-bipyridine ligands are more beneficial to enhancing the antibacterial activity of ruthenium complexes. While $[Ru(dmb)_2(BTPIP)](ClO_4)_2$ (**Ru(II)-3**) formed from 4,4′-dimethyl-2,2′-bipyridine showed the best bactericidal effect. Given that **Ru(II)-2** formed from 2,9-dimethyl-1,10-phenanthroline also showed lower MIC compared with **Ru(II)-1**, it is rational to believe that ruthenium complexes with better activity could be acquired by increasing the lipophilicity of auxiliary ligands.

Table 1 Minimum inhibition concentration (MIC) of all the compounds against *S. aureus* Newman bacterial strains

Compounds	Minimum inhibition concentration (MIC)
Ru(II)-1	0.050 mg mL^{-1}
Ru(II)-2	0.025 mg mL^{-1}
Ru(II)-3	0.003 mg mL^{-1}
Gentamicin	0.005 mg mL^{-1}
$RuCl_3{\cdot}3H_2O$	>0.250 mg mL^{-1}

Furthermore, the effects of ruthenium complexes on *S. aureus* growth were measured by growth curves. As shown in Fig. 1, the inhibitory effects of the three ruthenium complexes on *S. aureus* were dose-dependent. The growth of *S. aureus* was remarkably inhibited in the presence of the three ruthenium complexes (**Ru(II)-1**, **Ru(II)-2**, **Ru(II)-3**). Once again, there was scarcely any bacterial growth observed after incubation with the three ruthenium complexes at their minimum inhibitory concentration (MIC).

Next, the antibacterial activities of the three ruthenium complexes against Gram-negative pathogenic bacterium (*Pseudomonas aeruginosa*) were also investigated. The results indicated that the three compounds (**Ru(II)-1**, **Ru(II)-2**, **Ru(II)-3**) also showed antibacterial activity against *Pseudomonas aeruginosa* with MIC ranging from 0.15 to 0.25 mg mL^{-1} (Table S1†).

Many metal drugs which show significant antibacterial activity have been reported that could decrease cellular free thiols *via* ROS production.[23,24] Subsequently, a thiol detection assay kit was employed to determine intracellular free thiols level in *S. aureus* after **Ru(II)-3** treatment. The results suggested that **Ru(II)-3** treatment could decrease cellular free thiols in *S. aureus* and the mode of action of the compounds might include increased generation of intracellular reactive oxygen species (ROS) (Fig. S1†).

Inhibition of biofilm formation

It is well known that antibiotic resistance of bacteria is partly related to virulence factor secretion and biofilm formation. Bacteria could maintain stable growth in stressful environmental conditions through biofilm production. In fact, the biofilm mainly consists of exopolysaccharides (EPS), proteins and DNA and then adheres to abiotic surfaces.[25] In order to further investigate the antibacterial activity, the most active **Ru(II)-3** complex was further tested against biofilm formation. The drug concentration for biofilm formation assays was a sublethal concentration to ensure the inhibition of biofilm for-

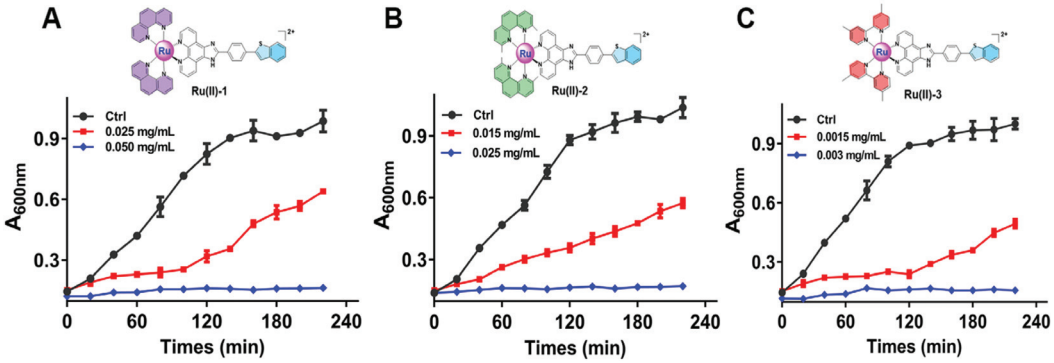


Fig. 1 The growth curve of *S. aureus* Newman strains in the presence of ruthenium complexes. The OD_{600} was recorded at 30 min intervals. (A) **Ru(II)-1**, (B) **Ru(II)-2**, (C) **Ru(II)-3**.

mation was not due to killing of the bacteria. In brief, after adding different concentrations of **Ru(II)-3** complex, *S. aureus* were transferred into a 24-well plate and further incubated at 37 °C for 36 h. The biofilm formation could be determined through crystal violet staining. As shown in Fig. 2, in the presence of 0.001 or 0.002 mg mL^{-1} of **Ru(II)-3**, the biofilm formation was significantly reduced by 10.5 and 25%, respectively. This indicated that ruthenium complexes could significantly reduce biofilm formation at sublethal concentration.

Ruthenium complexes showed synergism with the aminoglycoside antibiotics

The use of adjuvants is identified as an effective way to deal with bacterial resistance. As we had found that ruthenium complexes showed significant activity against *Staphylococcus aureus*, we wondered whether ruthenium complexes could also serve as adjuvants to enhance the action of existing antibiotics against *Staphylococcus aureus*. Therefore, the checkerboard method was performed to investigate the synergism between **Ru(II)-3** and common antibiotics against *S. aureus* as described.[20] All in all, we selected four commonly used antibiotics: ampicillin (β-lactam antibiotics); levofloxacin (quinolone antibiotic); gentamicin (aminoglycoside antibiotics); and chloramphenicol. Firstly, *S. aureus* Newman bacterial suspension (5×10^6 CFU mL^{-1}) with gradient concentrations of **Ru(II)-3** and selected antibiotics were added into each well of the 96-well plate. After incubation at 37 °C for 20 hours, the FICI values were calculated. The definition of FICI (fractional inhibitory concentration index) is the sum of the MIC values of each drug when used in combination divided by the MIC values of the drug when used alone. The synergy activity was defined by FICI values ≤0.5 while the antagonism was defined by FICI >4. As summarized in Fig. 3, the FICI values of the combination with **Ru(II)-3** and gentamicin was 0.187. This data indicated that **Ru(II)-3** exhibited synergism with gentamicin and **Ru(II)-3** might increase antibacterial activity of some aminoglycoside antibiotics against *S. aureus*.

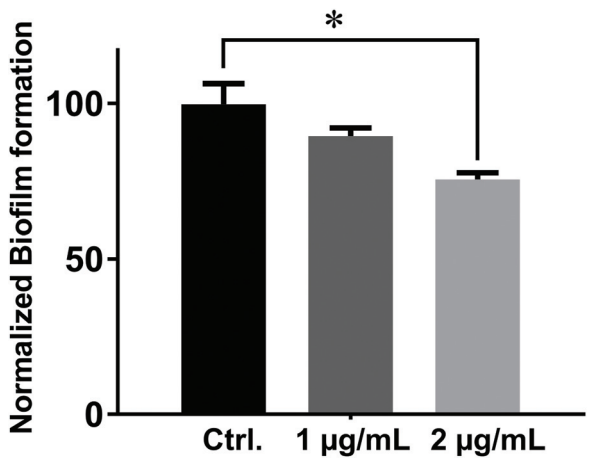
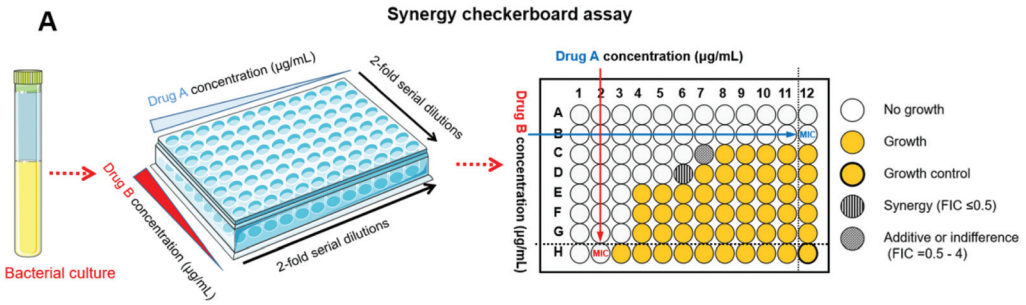


Fig. 2 The effect of **Ru(II)-3** on biofilm formation of *S. aureus* Newman strains. All experiments were performed with three biological replicates. The mean value of control groups is set as 100%.

Real-time qPCR

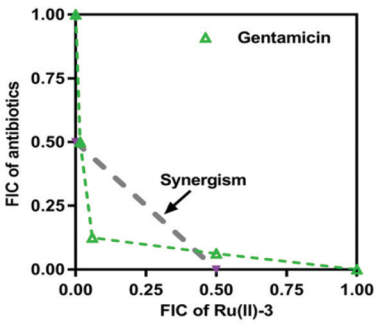
The checkerboard method confirmed that **Ru(II)-3** exhibited synergism with gentamicin against *S. aureus*. However, the mechanism of the observed synergetic effect is unknown. In our previous study, we found that bis(4-hydroxy-3-methylphenyl) sulfide (HMS) which could abrogate the regulatory function of *Sa*CcpA (catabolite control protein A from *Staphylococcus aureus*) also selectively increased the susceptibility of *S. aureus* to some aminoglycoside antibiotics.[26] Therefore, we speculated that the catabolite control protein A (*Sa*CcpA) might also serve as one target of **Ru(II)-3**. For further confirmation, real-time PCR was employed to investigate the transcription of the gene which CcpA regulated after **Ru(II)-3** treatment. As *Sa*CcpA mainly binds to the catabolite-responsive element (*cre*) sequences, we investigated the transcription of *citZ*, *hla* and *pckA* in the *S. aureus* strain after supplementation with **Ru(II)-3**. As shown in Fig. 4, the transcription levels of CcpA regulated genes (*citZ*, *ccpA* and *pckA*) were attenuated by **Ru(II)-3**. This phenomenon confirmed that **Ru(II)-3** restrained the gene regulatory activity of catabolite control protein A (CcpA) in *S. aureus*.



B

Type	Antibiotic	MIC (μg/mL)	FICI*
Aminoglycoside	Gentamicin	5	≤0.5
β-lactam	Ampicillin	0.5	=2
Quinolone	Levofloxacin	0.4	=2
Other	Chloramphenicol	10	=2

$FICI = FICI_A + FICI_B = A/MIC_A + B/MIC_B$



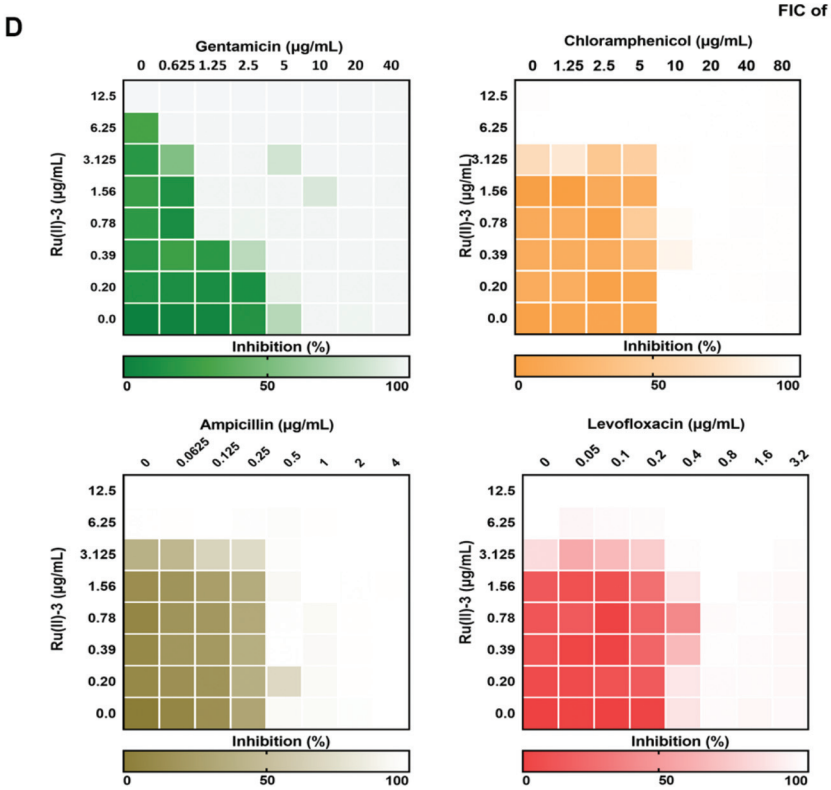


Fig. 3 The synergism between **Ru(II)-3** and four antibiotics was investigated by checkerboard assay (A). (B) The MIC values of four common antibiotics and the FICI values of the combination with **Ru(II)-3** against *S. aureus*. (C) The synergistic effects of **Ru(II)-3** with antibiotics were analysed by isobologram. (D) Heat plots of checkerboard assays for **Ru(II)-3** in combination with four antibiotics against *S. aureus* Newman.

Synergism activity investigated against *ccpA* mutant strains

The real-time qPCR data showed that **Ru(II)-3** exhibited selectivity synergism with the aminoglycoside antibiotics which might be related to the regulatory function of *Sa*CcpA being disturbed. For further confirmation, we performed the checkerboard method again to investigate the synergism between **Ru(II)-3** and aminoglycoside antibiotics against *S. aureus* Δ*ccpA* mutant (*ccpA* gene knockout). As we expected, the synergism between **Ru(II)-3** and aminoglycoside was completely

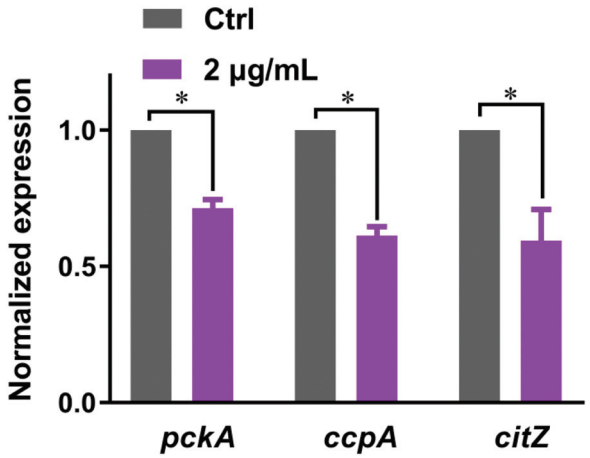


Fig. 4 The effect of **Ru(II)-3** on transcription of *pckA*, *ccpA* and *citZ* in *S. aureus*. All experiments were performed with three biological replicates. The mean value of control groups is set as 1. Results are shown as mean ± sd.

abolished in the *S. aureus ΔccpA* mutant (Fig. 5). Taken together, our results clearly demonstrated that **Ru(II)-3** exhibited selectivity synergism with the aminoglycoside antibiotics which might be by inhibiting the regulatory function of *Sa*CcpA. This also further accounts for the phenomenon that **Ru(II)-3** increased the susceptibility of *S. aureus* to aminoglycoside antibiotics.

In vivo bacterial infection treatment studies with mouse

The minimum inhibitory concentration assays and checkerboard method data all confirmed that ruthenium complexes functionalized with benzothiophene have good antimicrobial activity against *Staphylococcus aureus*. However, the *in vivo* antibacterial activity of ruthenium complexes is unknown. It has already been reported that ruthenium complexes would be rapidly cleared from the bloodstream after administration, so ruthenium complexes need to be suitable for topical application for surface infection treatment rather than injection routes.[27] Therefore, a mouse skin infection model was carried out in order to further confirm whether ruthenium complexes also possessed significant *in vivo* antibacterial activity. In brief, mice were inoculated with *S. aureus* Newman strains to develop abscesses on the skin. Subsequently, all the mice were divided into three groups and then the ointment containing **Ru(II)-3** (50 μg mL^{-1} or 100 μg mL^{-1}) was smeared onto the abscesses twice daily. After treatment for 64 hours, the abscess tissue was excised and homogenized for CFU quantification. The results are shown in Fig. 6, after treatment with **Ru(II)-3** ointment (100 μg mL^{-1}), smaller cutaneous abscesses were

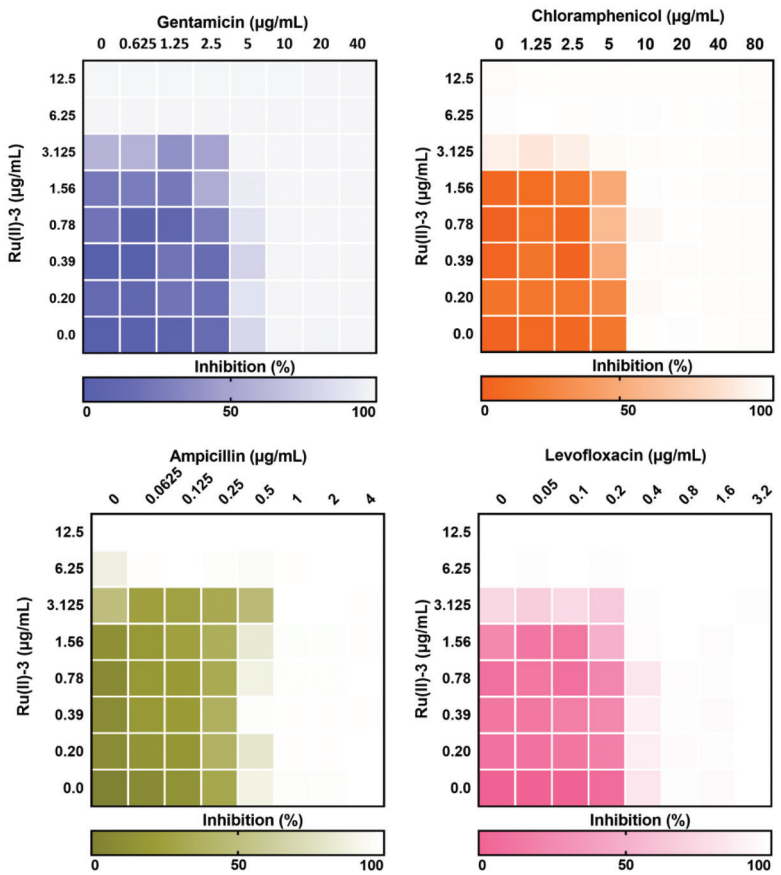


Fig. 5 Heat plots of checkerboard assays for **Ru(II)-3** in combination with different antibiotics against *S. aureus* ΔccpA mutant strains.

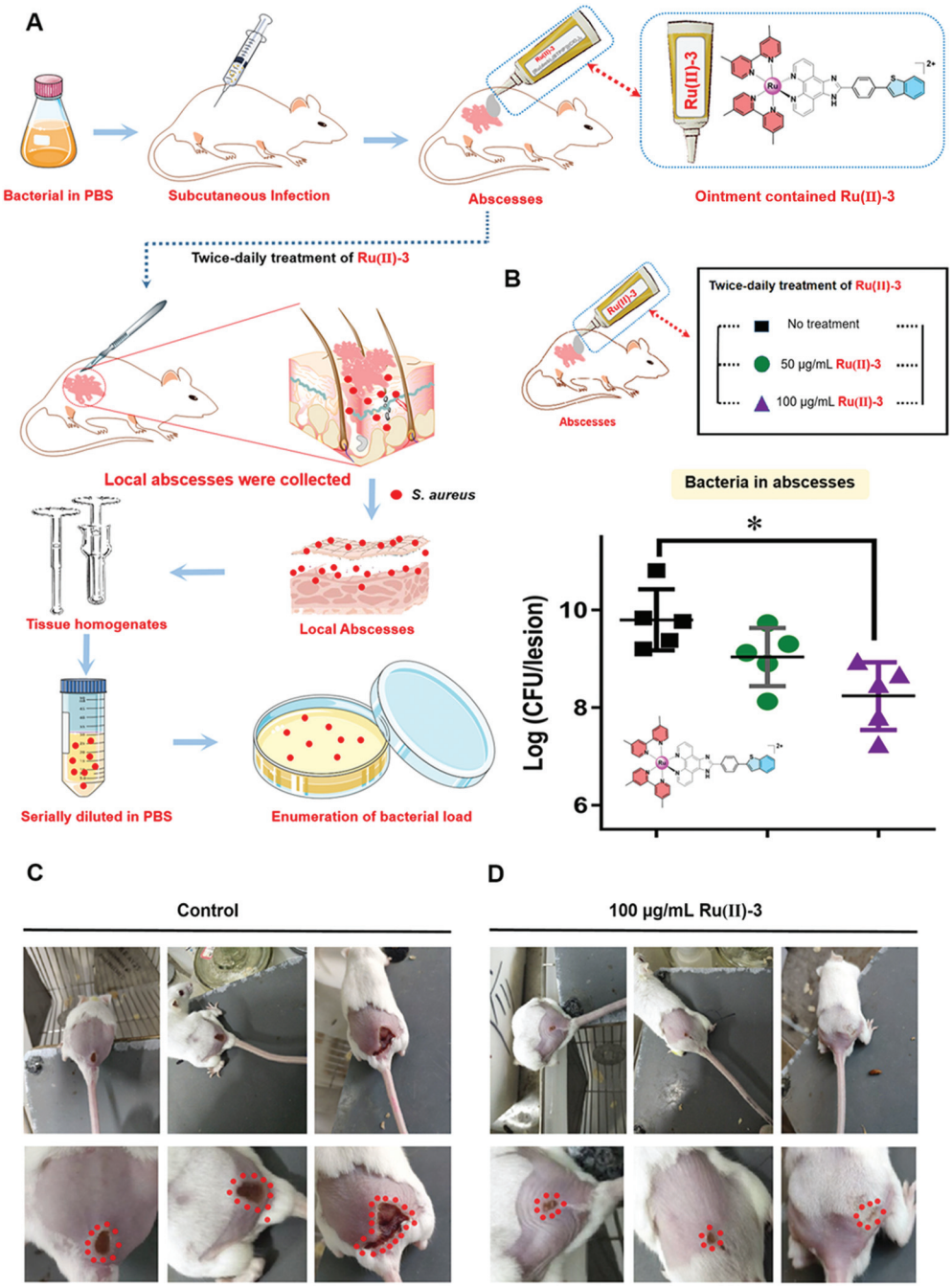


Fig. 6 The antibacterial activity of **Ru(II)-3** *in vivo* was studied with a murine skin infection model (A). (B) The bacterial load of local abscesses induced by *S. aureus* Newman was enumerated in control or **Ru(II)-3** treatment groups. The logCFU values are presented as the mean ± sd. The statistical difference is determined by a Mann–Whitney U test. (C and D) Representative photographs of the cutaneous abscesses in the presence/absence of **Ru(II)-3** therapy.

observed compared with the control group. In addition, the viable bacterial counts dropped significantly compared to the control group, with logCFU mean values of 9.8 (control) and 8.2 (**Ru(II)-3**, 100 μg mL^{-1}). This phenomenon suggested that **Ru(II)-3** indeed showed potential application for surface infection treatment.

Skin irritation test in mice

Ru(II)-3 showed potential application for surface infection treatment. Finally, to investigate the irritation effect of **Ru(II)-3** on the skin, the skin reaction was measured. In brief, ointment containing **Ru(II)-3** or distilled water control were softly

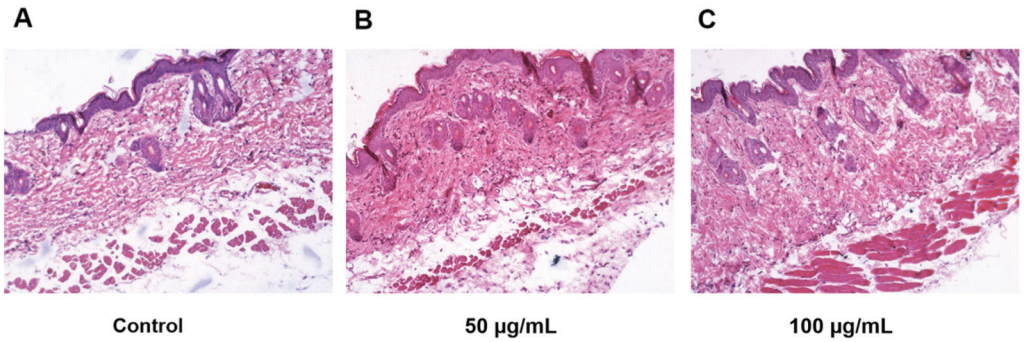


Fig. 7 The irritation effect of **Ru(II)-3** on the skin. (A) Control, 50 μg mL^{-1} (B) or 100 μg mL^{-1} (C) of **Ru(II)-3**. Representative histological results (hematoxylin and eosin (HE) stain of skin (Scale bar = 500 μm) are shown 4 days after attachment.

attached to the shaved part once a day for three days. On the fourth day the mice were killed by cervical dislocation and their shaved parts were removed and fixed in 4% paraformaldehyde at 4 °C for 1 day, then embedded in paraffin. Sequential sections were prepared for HE assays. As shown in Fig. 7, there were no appreciable skin reactions including the formation of erythema or edema during the experimental period, HE stain also confirmed this. Thus, **Ru(II)-3** could be considered to be a non-irritant material.

Conclusions

There is clearly a need for the development of new classes of antimicrobials to fight multidrug-resistant bacteria. In this study, three ruthenium complexes functionalized with benzothiophene were synthesized and their antimicrobial activities were assessed. Minimum inhibitory concentration (MIC) assays indicated that three of the ruthenium complexes (**Ru(II)-1**, **Ru(II)-2** and **Ru(II)-3**) all showed antibacterial activity against *S. aureus* and *P. aeruginosa*. The most active **Ru(II)-3** complex was also tested against biofilm formation and could significantly reduce biofilm formation at sublethal concentration. **Ru(II)-3** treatment could also decrease cellular free thiol in *S. aureus*. More importantly, a checkerboard assay indicated that **Ru(II)-3** also has potential as an antibiotic adjuvant because of **Ru(II)-3** could increase the antibacterial activity of some aminoglycoside antibiotics against *S. aureus*. In addition, the mechanism study clearly demonstrated that **Ru(II)-3** exhibited selectivity synergism with the aminoglycoside antibiotics which might be by inhibiting the regulatory function of *Sa*CcpA. Finally, *in vivo* bacterial infection treatment studies through a murine skin infection model and skin irritation test were also conducted. The results confirmed that ruthenium complexes functionalized with benzothiophene not only have good antimicrobial activity against *Staphylococcus aureus in vivo*, but also showed no irritation on the skin. However, the results are preliminary and will require further work to promote the design of better antimicrobial ruthenium agents. For example, the structure–activity relationship indicated that ruthenium complexes with better activity could be acquired by increasing the lipophilicity of the auxiliary ligands. This will prompt us to design better organic ligands to improve the antibacterial activity of ruthenium complexes in our next work. In addition, the synergy observed in our study provides an interesting starting point for further investigations.

Ethics statement

The animal study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985) and was reviewed and approved by Institutional Animal Care and Use Committee of Guangxi Normal University (Guilin, China).

Conflicts of interest

The authors have declared that there is no conflict of interest.

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