The development of three ruthenium-based antimicrobial metallodrugs: Design, synthesis, and activity evaluation against Staphylococcus aureus

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Abstract
The development of new classes of antimicrobial is urgently needed due to the widespread occurrence of multi-resistant pathogens. In this study, three novel ruthenium complexes: [Ru(dmob)2(BTPIP)][PF6]2 (Ru(II)-1), [Ru(dbp)2(BTPIP)][PF6]2 (Ru(II)-2), and [Ru(dpa)2(BTPIP)][PF6]2 (Ru(II)-3) (dpa = 2,2’-dipyridylamine, dmob = 4,4’-dimethoxy-2,2’-bipyridyl, dbp = 4,4’-di-tert-butyl-2,2’-dipyridyl, BTPIP = 4-(benzo[b]thiophen-2-yl)phenyl-1H-imidazo[4,5-f][1,10]phenanthroline) are synthesized and investigated as antimicrobial metallodrugs. We demonstrate that all three complexes have significant antimicrobial activity against Staphylococcus aureus by testing their minimal inhibitory concentrations = 0.0015–0.0125 mg/mL. The antibacterial activity of the best active complex Ru(II)-3 is 13 times that of ofloxacin (minimal inhibitory concentration = 19.5 μg/mL). Importantly, Ru(II)-3 not only increases the susceptibility of Staphylococcus aureus to existing common antibiotics but also shows noticeably delayed and decreased resistance in Staphylococcus aureus since the minimal inhibitory concentration values of Ru(II)-3 only increased eightfold times after 20 passages. Furthermore, the biofilms formation and rabbit erythrocyte hemolysis assays verified that Ru(II)-3 also efficiently inhibit the biofilm formation and toxin secretion of Staphylococcus aureus.

Keywords
antimicrobial activity, ruthenium complexes, Staphylococcus aureus, metallodrugs

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Introduction
As a major bacterial human pathogen, Staphylococcus aureus (S. aureus) can lead to many life-threatening infections. The overuse of antibiotics leads to the emergence of methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Staphylococcus aureus (VRSA).1–5 The proliferation of these multi-resistant pathogens poses a significant threat to human health.6 In addition, only six new classes of antibiotics have been approved in the past 20 years.7,8 Thus, it is important to develop new classes of antimicrobial or antimicrobial adjuvants to respond to this crisis.

Metallodrugs play an important role in the development of anticancer agents.9 As a matter of fact, metal-based complexes exhibit significant antimicrobial activity against multiple bacteria. For example, bismuth citrate is used for the treatment of gastric ulcers to eradicate Helicobacter pylori, while silver sulfadiazine remains the best choice for the treatment of bacterial infection after burns.10 It is reported that ruthenium-based complexes also display potent antimicrobial activity against human pathogens.11–13 A Ru-carbonyl precursor was reported to show a minimum 13 times that of ofloxacin (minimal inhibitory concentration = 19.5 μg/mL).
inhibitory concentration (MIC) at a nanogram level per milliliter, which renders it a novel antimicrobial polymer. In addition, ruthenium complexes have potential as novel therapeutic candidates to treat *S. aureus* infections via inhibition of biofilm formation. More interestingly, ruthenium complexes containing pyridine monodentate ligands show significant photocatalyzed antibacterial activity against many drug-resistant bacteria including MRSA and vancomycin-resistant *Enterococcus* (VRE).

It has been reported that benzothiophene-based organic molecules and benzothiophene-conjugated compounds exhibit strong inhibitory effects against *S. aureus*. In previous studies, we introduced benzothiophene to ruthenium complexes and synthesized four ruthenium complexes with MIC values against *S. aureus* ranging from 0.003 to 0.050 mg/mL. In addition, these complexes also showed moderate inhibitory activity against proliferation of A549 cells. More importantly, structural-activity relationship studies clearly indicated that complexes containing 2,2'-bipyridine ligands exhibited better antibacterial effects and the presence of a methyl group on the auxiliary ligands was also helpful in enhancing the antibacterial activity of the ruthenium complexes. Therefore, we were interested in studying the effects of other bipyridine derivatives on the antibacterial activity of benzothiophene-based ruthenium complexes. In the present, we have designed three complexes based on bipyridine derivatives and benzothiophene-substituted ligands with the aim of developing novel ruthenium complexes with improved antibacterial activity (Scheme 1(a)). Three ruthenium complexes: [Ru(dmob)2(BTPIP)](PF6)2 (Ru(II)-1), [Ru(dpdb)(BTPIP)](PF6)2 (Ru(II)-2) and [Ru(dpa)(BTPIP)](PF6)2 (Ru(II)-3) (dpa = 2,2'-dipyridylamine, dmob = 4,4'-dimethoxy-2,2'-bipyridyl, dpdb = 4,4'-di-tert-butyl-2,2'-dipyridyl, and BTPIP = 4-(benzo[2,1-b:7,6-b']dithiophene-2-yl)phenyl-1H-imidazo[4,5-f][1,10]phenanthroline) were synthesized (Scheme 1(b)) and characterized by IR, 1H NMR, and high-resolution mass spectrometry (HRMS). Their antimicrobial effects against *S. aureus* were examined through investigations of the MICs and the growth curves of bacteria. To further investigate their antimicrobial effects, the ability of Ru(II)-3 to inhibit biofilm formation and toxin secretion from *S. aureus* as also tested. In addition, the development of bacterial resistance in the presence of Ru(II)-3 was also explored. Finally, the synergism between Ru(II)-3 and common antibiotics against *S. aureus* was examined to verify whether Ru(II)-3 has potential as an antimicrobial adjuvant.

**Results and discussion**

**Chemistry**

The BTPIP and the corresponding three ruthenium complexes were synthesized in an identical manner to that previously described by us. The structures of the ligands and ruthenium complexes were successfully characterized by NMR, HRMS, and IR. For the HRMS spectra, all the found signals of the [M-2PF6]2+ ions were in complete agreement with the theoretical value.

**Antibacterial activity studies**

Initially, a microbroth dilution experiment was performed to determine the MIC of the three complexes against *S. aureus* and the results are presented in Table 1. In addition, the MIC values are compared with the data for ruthenium complexes 4 (Scheme 1(a)), which we previously prepared published to understand the structure-activity relationships. In fact, all three compounds functionalized with benzothiophene showed very low MIC values ranging from 1.5 to 12.5 μg/mL against *S. aureus*. To our surprise, Ru(II)-2 containing a 4,4'-dibutyl-2,2'-dipyridyl unit (MIC = 12.5 μg/mL) showed relatively higher MIC values compared to the ruthenium complex 4 with 4,4'-dimethyl-2,2'-dipyridyl (MIC = 3 μg/mL) (Scheme 1(a)). In contrast, the highest activities were identified for Ru(II)-3 incorporating 2,2'-dipyridylamine ligands (MIC = 1.5 μg/mL). It worth noting that the antibacterial activity of Ru(II)-3 was 13 times that of ofloxacin (MIC = 19.5 μg/mL). Furthermore, the MIC values for Ru(II)-3 decreased onefold compared with the highest activities compound 4. The MIC values of Ru(II)-I containing a 4,4'-dime thoxy-2,2'-bipyridyl ligand were lower than of Ru(II)-2; hence, we speculated that the reason for this phenomenon might be that the substituent groups with greater steric hindrance on dipyridyl weaken the binding of the complexes to the targeted biomacromole.

Next, we monitored the absorbance of bacteria at 600 nm every 30 min and plotted the growth curve of *S. aureus* after adding the three complexes. The curves showed that *S. aureus* growth was obviously inhibited at 0.5 × MIC values and completely suppressed at the MIC values of three compounds, respectively (Figure 1).

**Biofilm formation inhibition by Ru(II)-3**

Many pathogenic bacteria form biofilms to avoid attack by drug molecules. Bacterial aggregate membrane-like objects form when bacteria adhere to a contact surface, these bacteria can then clump together and form a biofilm which helps bacteria survive in harsh environments. The bacteria inside the biofilm have a stronger resistance to antibiotics and this process can also result in the bacteria developing resistance in the case of drug treatment. Therefore, we speculated that if the complexes can inhibit the biofilm formation of *S. aureus*, they might also effectively prevent the bacteria from developing drug resistance. Subsequently, we monitored the ability of Ru(II)-3, which showed the highest activities, to inhibit *S. aureus* forming a biofilm. We detected biofilm formation mainly through staining the biofilm by crystal violet. The details of the experiment as well as the results are illustrated in Figure 2. The amount of stained biofilm decreased by 29.6% on treatment with 0.375 μg/mL of Ru(II)-3, while biofilm formation was...
reduced further by 62.4% in the presence of 0.75 μg/mL of \textit{Ru(II)}-3. These results prove that \textit{Ru(II)}-3 can prevent \textit{S. aureus} from developing biofilms.

Studies on the development of \textit{S. aureus} resistance to ruthenium complexes

Since the complex \textit{Ru(II)}-3 remarkably inhibits the formation of bacterial biofilms, we assume that the \textit{S. aureus}
cannot easily develop resistance to Ru(II)-3. To further verify this, we performed the drug-resistance develop experiments to investigate the ability of S. aureus to develop resistance in the presence of Ru(II)-3. In brief, the S. aureus bacteria were repeatedly exposed to 0.5-fold minimal inhibition concentration of 20 successive runs to induce the bacteria to develop resistance (Figure 3(a)). The change of the MIC against S. aureus of Ru(II)-3 and ampicillin was shown in Figure 3(b). As can be seen, the MIC values of Ru(II)-3 increased only eightfold times after 20 runs. As a comparison, the MIC values of ampicillin increased over 1300 times. This verified that S. aureus could not easily develop resistance to Ru(II)-3.

**Inhibition of hemolysin secretion by the Ru(II)-3**

Besides directly killing bacteria with antibiotics, a novel antibacterial strategy that involves restraining the toxin secretion of bacteria has received more and more
attention in recent years. The advantage of this strategy might reduce or completes avoid the development of drug resistance. In *S. aureus* strains, α-hemolysin (Hla) is an important extracellular toxin that plays an important role in *S. aureus* infections by embedding into the human cell membrane and leading to cell damage. Thus, we investigated the hemolysin secretion of *S. aureus* after incubation with the Ru(II)-3. In brief, the hemolysin levels in bacterial culture supernatants were determined by adding bacterial culture supernatants to rabbit blood cells. To ensure the inhibition of hemolysin toxin secretion was not due to killing of the bacteria, the concentration of Ru(II)-3 was sublethal and the bacterial growth was not affected (Figure 4(b)). The results (Figure 4(c) and (d)) clearly show that the hemolysin activity of the *S. aureus* culture supernatants decreased remarkably as the amount of red blood cell fragmentation was reduced by 18.6% (0.375 μg/mL Ru(II)-3) and 42.4% (0.75 μg/mL Ru(II)-3) compared to the control group. This proved that the ruthenium complex could effectively inhibit the hemolysin toxin secretion of *S. aureus*.

**Figure 3.** The bacterial resistance development experiment (a). The change of the minimum inhibitory concentration of Ru(II)-3 or ampicillin against *S. aureus* (b). This experiment was performed with three biological replications.

**Figure 4.** The effect of Ru(II)-3 (a) on the hemolysin toxin secretion of *S. aureus*. The OD_{600} values of *S. aureus* culture suspension (b), and the hemolysin activity of *S. aureus* culture supernatants (c and d) after incubation with 0.375 or 0.75 μg/mL of Ru(II)-3. All experiments were performed with three biological replications.
The synergism between Ru(II)-3 and common antibiotics

Finally, the checkerboard method assay (Figure 5(a)) was employed to further explore whether ruthenium complexes have potential as antimicrobial adjuvants. The synergism between ruthenium complexes and antibiotics can be confirmed from the fractional inhibitory concentration index (FICI). FICI values less than 0.5 after combination indicate that there is a synergistic effect. In general, we selected four common antibiotics. The heat plots of the combination experiment (Figure 5(b)) and the corresponding FICI values (Figure 6) clearly confirm that Ru(II)-3 significantly increases the antibacterial activity of gentamicin (FICI of 0.16) and tobramycin (FICI of 0.28) against S. aureus. These results also demonstrate that Ru(II)-3 has potential value as an antimicrobial adjuvant.

Conclusion

In conclusion, three novel ruthenium complexes ([Ru(dmob)2(BTPIP)](PF6)2 (Ru(II)-1), [Ru(dbp)2(BTPIP)](PF6)2 (Ru(II)-2), [Ru(dpa)(BTPIP)](PF6)2 (Ru(II)-3)) based on our previously studies were designed and successfully synthesized in this study. Investigations of their biological activity research confirmed that all three complexes could completely suppress growth of the pathogenic bacteria S. aureus at microgram concentrations. The structure-activity relationship studies indicated that substituent
groups with greater steric hindrance on the ruthenium complexes reduced their antimicrobial activity. Further research on the antibacterial activity of the most activate complex Ru(II)-3 indicated that biofilm formation and toxin secretion from S. aureus were inhibited by a subinhibitory concentration of Ru(II)-3. More importantly, Ru(II)-3 noticeably delayed and decreased resistance in S. aureus, and also increased the susceptibility of S. aureus to existing common antibiotics. Overall, this work raises the possibility of developing ruthenium complexes containing benzothiophene-substituted ligands as potential antibacterial metallodrugs or antimicrobial adjuvants against S. aureus. In addition, the efficacy of Ru(II)-3 against other pathogenic bacteria merits further exploration.

Material and methods

**Synthesis of BTPIP**

This ligand was synthesized via the previously reported route.21

**Synthesis of [Ru(dmob)₂(BTPIP)](PF₆)₂ (Ru(II)-1)**

A mixture of BTPIP (42.6 mg, 0.1 mmol), Ru(dmob)₂Cl₂·2H₂O (60.4 mg, 0.1 mmol) and ethylene glycol (10 mL) in a 50-mL reaction flask and was heated at 150 °C under argon and refluxed for 8 h. The resulting red reaction solution was cooled to room temperature and potassium hexafluorophosphate was added to give a red precipitate. The resulting precipitate was filtered off and washed with water. Finally, the crude product was absorbed onto the stationary phase and then further purified using a neutral alumina column with xylene/acetonitrile (5:1, v/v) as eluent. A red powder was obtained after removal of the solvent under reduced pressure. Yield: 86.58 mg, 45%. IR (KBr): 578, 726, 726, 1156, 1161, 3433 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆): δ 9.10 (d, J = 9.8 Hz, 2H), 8.97 (s, 1H), 8.49 (t, J = 17.2 Hz, 2H), 8.04 (dd, J = 6.7 Hz, 2H), 7.93 (dd, J = 5.2 Hz, 2H), 7.66 (d, J = 7.4 Hz, 4H), 7.49 (dd, J = 17.4, 7.3 Hz, 4H), 7.37 (d, J = 6.7 Hz, 2H), 1.45 (s, 18H), 1.36 (s, 18H); HRMS (ESI): m/z: calcld for [M-2PF₆-H]⁺ C₆₃H₆₃N₈RuS, 1065.39292; found: 1065.39344.

**Biology**

**Antibacterial activity.** The MIC of all complexes against S. aureus was determined by the multiple dilution method. The S. aureus Newman strain was cultured in Tryptone soybean broth medium (TSB) medium overnight, and then diluted with fresh TSB medium (1:1000). After adding 50 μL of the ruthenium complex to a 96-well plate with a gradient concentration, 200 μL of bacterial suspension was added. The plate was incubated for 20 h at 37 °C in order to

| Type       | Antibiotic   | MIC (µg/mL) | FICI* |
|------------|--------------|-------------|------|
| Aminoglycoside | Gentamicin    | 0.98        | 0.16 |
| Aminoglycoside | Tobramycin   | 0.98        | 0.28 |
| Lincomamide  | Clindamycin  | 15.6        | >0.5 |
| Quinolone    | Ofloxacin    | 1.95        | >0.5 |

FICI* = FICIₐ + FICIₐₐ = A/MICₐ + B/MICₐ

Figure 6. The FICI values of four antibiotics after combination with Ru(II)-3 against S. aureus (a). The isobologram indicates the synergistic effects of Ru(II)-3 with two antibiotics (b).
determine the MIC values. To plot the growth curve of bacteria, the overnight-cultured bacteria were diluted 100 times with fresh TSB medium, and then the ruthenium complex was added. The bacterial suspension was cultured in a constant temperature incubation shaker and the OD value was recorded every half an hour.

Biofilm formation assay

An overnight culture of *S. aureus* was diluted 1000-fold with fresh TSB medium. A 24-well microtiter plate was filled with 2 mL of bacterial suspension with or without the ruthenium complex. After incubation at 37°C for 48 h, the suspended bacteria were removed and the plate was washed three times with Phosphate Buffer Saline (PBS). Subsequently, the plate was dried at room temperature and the biofilm was stained for 5 min using crystal violet. After removal of crystal violet and further washing with PBS until the water layer was clear, glacial acetic acid (2 mL) was added and the absorbance was monitored at 595 nm.

Hemolysin activity assay

Overnight cultured bacteria were diluted 1000 times by fresh TSB medium to obtain the bacterial suspension. After adding the ruthenium complex, the bacterial suspension was incubated at 37°C and centrifugal at 220 r/min for 16 h. The bacterial supernatant could be prepared by centrifugation (12,000g, 5 min). Subsequently, 150 μL of bacterial supernatant together with 25 μL of rabbit blood cells were added to 1 mL of Bovine serum albumin (BSA) buffer. The solution was incubated at 37°C for 30 min and the supernatant was obtained by centrifugation. The optical density of the supernatant was measured at 543 nm.

Checkerboard assay

Mixture of antibiotics (50 μL) (gentamicin, tobramycin, clindamycin, or ofloxacin) and Ru(II)-3 of gradient concentration were added to a 96-well plate. After added 200 μL of the *S. aureus* suspension, the plate was incubated for 20 h at 37°C. The FICI could be obtained from the MIC of the antibiotic and Ru(II)-3, respectively. The isobolograms were plotted using the GraphPad Prism software.

Bacterial resistance development

A 96-well polystyrene plate was used to explore the resistance development of *S. aureus* in the presence of the ruthenium complex or antibiotic. In short, the bacteria were cultured in TSB medium at 37°C overnight, and then the MIC of the ruthenium complex was determined. Taken 10 μL bacteria suspension from the well of 0.5 × MIC into fresh TSB medium and then incubated for another 6 h. Subsequently, the MIC values of the ruthenium complex were measured again. Repeat the above procedure 20 times and record the change of MIC values.

Author contributions

X.L. participated in the data analysis, the design of the study and drafted the manuscript. B.H. and X.D. conceived and designed the study and critically revised the manuscript. L.W., Y.X., and S.Z. carried out the work and helped to perform the experiments and analyze the data. G.J. and J.W. provided the experimental guidance. All authors provided the final approval for publication and agree to be held accountable for the work performed therein.

Declaration of conflicting interests

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Data accessibility

The data that support the findings of this study are available from the corresponding author upon request.

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