

SHORT COMMUNICATION

Diastereomeric bactericidal effect of Ru(phenanthroline)₂dipyridophenazine

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ABSTRACT

Metal susceptibility assays and spot plating were used to investigate the antimicrobial activity of enantiopure [Ru(phen)₂dppz]²⁺ (phen = 1,10-phenanthroline and dppz = dipyrido[3,2-*a*:2',3'-*c*]phenazine) and [μ-bidppz(phen)₄Ru₂]⁴⁺ (bidppz = 11,11'-bis(dipyrido[3,2-*a*:2',3'-*c*]phenaziny)), on Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* as bacterial models. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined for both complexes: while [μ-bidppz(phen)₄Ru₂]⁴⁺ only showed a bactericidal effect at the highest concentrations tested, the antimicrobial activity of [Ru(phen)₂dppz]²⁺ against *B. subtilis* was comparable to that of tetracycline. In addition, the Δ-enantiomer of [Ru(phen)₂dppz]²⁺ showed a 2-fold higher bacteriostatic and bactericidal effect compared to the Λ-enantiomer. This was in accordance with the enantiomers relative binding affinity for DNA, thus strongly indicating DNA binding as the mode of action.

KEYWORDS

antimicrobial activity, chirality, confocal microscopy, emission, metal susceptibility assay, minimum bactericidal concentration, minimum inhibitory concentration, ruthenium complex

1 | INTRODUCTION

The treatment of bacterial infections has become more and more problematic due to the emergence of multidrug-resistant pathogens.^{1,2} With few exceptions, the antibiotics that are currently available for clinical use all target the same limited set of bacterial components (the cell wall, the cell membrane, and a few enzymes essential for bacterial growth), thus increasing the risk for multiresistance.³ Another problem is that new antibiotics that are coming onto the market are mostly variants of existing drugs, with the risk that resistance mechanisms have already developed.⁴ A noteworthy exception is the recent discovery of teixobactin, the first member of a novel class of antibiotics that targets lipid II and lipid

III, both membrane-anchored cell wall precursors essential for bacterial cell wall synthesis.⁵

There are several components in bacteria that have not been fully explored as potential antimicrobial targets. Bacterial DNA is an attractive antimicrobial target, provided that specificity to bacterial cells can be established, as DNA targeting compounds have the potential risk of also damaging eukaryotic cells. Actinomycin D, doxorubicin, and daunorubicin are a few examples of DNA binding antimicrobials that were deemed too cytotoxic for the host⁶ and were therefore developed as anticancer treatments.^{7–9}

While a number of reports have analyzed the combination of transition metals with antibiotics as a way to increase compounds' potency against infections

(“metalloantibiotics”),^{10–12} much fewer studies have tested the antimicrobial activity of metal complexes alone. Platinum-containing complexes such as cisplatin were shown to have antimicrobial activity, but were too damaging to eukaryotic cells, thus only suitable for anticancer treatment.^{13,14} Ruthenium complexes show less general toxicity than platinum compounds,^{15,16} and in particular ruthenium(II) polypyridyl complexes have several properties that would make them suitable for antimicrobial treatments (e.g., water soluble, coordinately saturated, and inert to substitution). While the DNA intercalating ability of chiral ruthenium(II) polypyridyl complexes have been extensively studied and debated for more than 30 years,^{17–21} there has been comparably little interest in testing their antimicrobial activity despite the pioneering work of Dwyer et al. more than 60 years ago.^{22,23} This is somewhat surprising, as they are very stable, readily synthesized, and have strong DNA binding ability.²⁴ In addition, their properties are readily modulated by the peripheral ligands, making it possible to influence DNA binding and enantioselectivity.²⁰ While the antimicrobial activity of mononuclear ruthenium(II) polypyridyl complexes has previously been demonstrated,^{25,26} to the best of our knowledge no reported study resolved the complexes in their pure enantiomeric forms. Even fewer studies have been published on antimicrobial activity in binuclear ruthenium(II) polypyridyl complexes, with the exception of the work by Li and Keene et al.^{27–33} By coordinating bidentate ligands to the ruthenium ion two conformations are possible: either a right- (Δ) or left-handed (Λ) helical structure. The resulting diastereomeric interactions when bound to a chiral macromolecule, such as

DNA, would assumingly not be identical for both enantiomers.

In this study we analyzed the antimicrobial activity of enantiomeric pure $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ (Ru-phen) (phen = 1,10-phenanthroline and dppz = dipyrido[3,2-*a*:2',3'-*c*]phenazine) and $[\mu\text{-bidppz}(\text{phen})_4\text{Ru}_2]^{4+}$ (biRu-phen) (bidppz = 11,11'-bis(dipyrido[3,2-*a*:2',3'-*c*]phenazinyl)) (see Scheme 1). Both complexes are well-established DNA intercalators and Ru-phen has already been shown to not be cytotoxic against HeLa cells.³⁴ As bacterial models, we used Gram-negative *E. coli* and Gram-positive *B. subtilis*. As a reference for antimicrobial activity, we used tetracycline, a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria.

2 | MATERIALS AND METHODS

2.1 | Materials

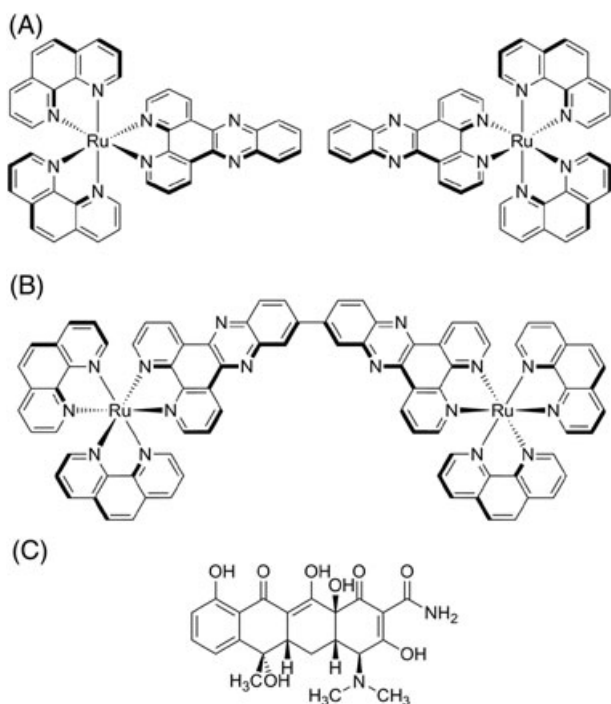
Enantiopure $[\text{Ru}(\text{phen})_2\text{dppz}]\text{Cl}_2$ and $[\mu\text{-bidppz}(\text{phen})_4\text{Ru}_2]\text{Cl}_4$ used in this study were synthesized as described elsewhere.^{35,36} Concentrations were determined spectrophotometrically using extinction coefficients: $\epsilon_{440} = 20\,000\ \text{M}^{-1}\ \text{cm}^{-1}$ for Ru-phen and $\epsilon_{262} = 200\,000\ \text{M}^{-1}\ \text{cm}^{-1}$ for biRu-phen. Tetracycline hydrochloride (Sigma-Aldrich, St Louis, MO), the DNA stain Hoechst 33342 (bis-benzimidazole, Thermofisher, Waltham, MA), PFA (para-formaldehyde, Sarstedt, Sweden), SDS (sodium dodecyl sulfate, Sigma-Aldrich) and other chemicals were used without purification. Luria-Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl), 0.9% saline solution, 10 mM NaPO_4 + 1% LB medium (pH 7), LB agar plates and phosphate-buffered saline (PBS) were prepared at the department of Clinical Microbiology at the Sahlgrenska University Hospital.

2.2 | Bacterial Strains and Culture Conditions

The bacterial strains used in this study were *Escherichia coli* DSM 1103 and *Bacillus subtilis* 168. The strains were purchased from the American Type Culture Collection (ATCC, Manassas, VA) (ATCC 25922, ATCC 23857) and activated from freeze-dried conditions in accordance with the protocol provided upon delivery. Strains were grown on LB medium aerobically at 37°C. Stock solutions of the bacterial strains were prepared from freshly grown bacterial cultures and stored in 20% glycerol at -70°C .

2.3 | Stock Solutions of Tetracycline and Metals

A stock solution of 5 mg/ml tetracycline was prepared in 70% ethanol and stored protected from light at 8°C. Stock solutions of the complexes were prepared at 5 times the highest concentration used in the challenge plate ($\sim 2000\ \mu\text{M}$) by dissolving the chloride salts in autoclaved MilliQ water (Billerica, MA).



SCHEME 1 Structures of (a) Λ - (left) and Δ Ru-phen (right), (b) $\Lambda\Lambda$ biRu-phen, and (c) tetracycline

2.4 | Metal Susceptibility Assays

Metal susceptibility assays were performed using a method similar to the MBEC high-throughput assay as previously described.³⁷ Frozen stocks of the bacterial strains were streaked out on agar LB plates to obtain subcultures. After 24 h incubation, a single colony from each strain was collected from the subcultures and suspended in LB medium. After 24 h incubation, this suspension was diluted 100-fold in LB medium and left to grow an additional 4 h. To ensure that the bacterial growth was in the exponential phase, optical density (OD) at 650 nm was measured and the bacterial solutions were spot plated for colony count (OD = 0.3, CFU/ml = 10⁸, diluted 1:3, used 20 µl so that CFU/well = 2 × 10⁶). Serial dilutions of the ruthenium complexes were made in LB medium along the length of a sterile 96-well microtiter plate (the challenge plate), allowing the first column to serve as a sterility control and the last column to serve as a growth control. Each ruthenium complex and tetracycline were tested in triplicate and repeated once to ensure reproducibility. The challenge plates were incubated for 24 h. To ensure aerobic conditions, a shaking table was used for incubation of the plates and cultures. A schematic illustration summarizing the assay protocol can be found in the Supporting Information (Figure S1).

2.5 | Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The MIC is defined as the lowest concentration of a compound that inhibits the visual growth of an organism. We determined the MIC values by reading the optical density of the challenge plate at 650 nm (OD₆₅₀) on a SpektraMax Plus 384 Microplate Reader (Molecular Devices, MSD Analytical Technologies, UK). The cellular suspensions in each row of the challenge plate were then transferred to new sterile 96-well microtiter plates and diluted 10¹–10⁸-fold with 10 mM NaPO₄ + 1% LB medium. Twenty-µl aliquots of each dilution of each bacterial culture were spot plated in duplicate onto LB agar plates and incubated for 24 and 48 h. The MBC is defined as the lowest concentration of an antibiotic required to kill a particular bacterial strain. The MBC values were determined by qualitatively scoring the spot plates for bacterial growth (this was done after 24 and 48 h to ensure complete eradication of the bacterial cells). The number of viable bacteria was expressed in CFU (colony-forming units) per ml:

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{Average colony count}}{\text{Volume}} \times \text{dilution} \quad (1)$$

2.6 | DNA Binding Emission

Emission spectra were recorded on a Varian Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA). Inoculums of *B. subtilis* and *E. coli* in

the exponential phase were incubated overnight (37°C, 120 rpm), either with the enantiomers of Ru-phen and biRu-phen (40 µM) in LB medium or in the medium alone. After incubation, 1 ml of each bacterial suspension was centrifuged at 6000 g at room temperature. The pellet was washed twice in 1× PBS and then resuspended in 1 ml of 1× PBS solution. The samples were excited at 440 nm and emissions were recorded at 500–800 nm.

2.7 | Confocal Microscopy

Confocal microscopy images were obtained with a Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Germany) controlled by Leica Application Suite Advanced Fluorescence (LAS AF) 2.6.0.7266 software. Inoculums of *B. subtilis* and *E. coli* in the exponential phase were incubated with Δ- and ΔRu-phen (40 µM) in LB medium overnight (37°C, 120 rpm) together with nonexposed bacterial suspensions as controls. After incubation, 1 ml of each bacterial suspension was centrifuged at 6000 g at room temperature. The pellet was washed twice in 1× PBS and then resuspended in 1 ml of 1× PBS solution. Droplets (40 µl) of each suspension were placed on microscopy glass slides (Superfrost, VWR), allowed to adhere for 10 min, and then fixed with 2% PFA in PBS for 10 min. The slides were washed using PBS solution and the bacteria were stained with Hoechst 33342 (1:10,000 dilution) for 15 min. The slides were washed one final time with PBS before mounting using fluorescence mounting medium (Invitrogen, La Jolla, CA). Samples were viewed using a 63× objective (total magnification 630×) and Ru-phen (λ_{ex} ~ 440 nm, λ_{em} ~ 610 nm) was excited using a 458 nm blue argon laser at 40% intensity. Emission fluorescence of Ru-phen was collected at 600–670 nm. Hoechst 33342 (λ_{ex} ~ 354 nm, λ_{em} ~ 486 nm) was excited with an ultraviolet diode laser and detected using a blue/cyan filter.

2.8 | Nucleotide Leakage

Measurements were performed in accordance with a previous method described by Henie et al., with minor modifications.³⁸ Solutions containing bacterial inoculum in the exponential phase treated with biRu-phen (~300 µM) were filtered through a 0.22-µm pore size Millipore Express Millex GP sterile syringe filter with PES membrane (Merck Millipore, Germany) at 0, 10, 20, 30, 40, 50, and 60 min; 3% SDS solution was used as a comparison. Absorption at 260 nm for bacterial nucleic acids were measured using a Varian Cary 4000 UV/vis spectrophotometer (Agilent Technologies). All measurements were performed in triplicate and repeated once to ensure reproducibility.

3 | RESULTS AND DISCUSSION

In this study we investigated whether there was an enantiomeric difference in antimicrobial activity of both

mononuclear and binuclear dppz-ruthenium(II)-centered complexes with phen as the peripheral ligands, and the plausible mode of action for their antimicrobial effect.

Neither $\Delta\Delta$ - nor $\Lambda\Lambda$ biRu-phen showed any significant antimicrobial activity against either bacterial strain, and no enantiomeric difference could be observed. The sudden drop in bacterial growth at the highest complex concentrations could be caused by lysis of the cellular membrane, and not by interaction with DNA. To verify this hypothesis, we exposed microbial cells to the highest concentration of binuclear complexes and quantified the release of nucleic acids. During 60 min of biRu-phen exposure the optical density at 260 nm of cell-free filtrates increased for both *B. subtilis* and *E. coli*, indicating nucleotide leakage (see

Fig. S2 in the Supporting Information). Notably, the leakage of nucleic acid material was similar for the binuclear complexes and a 3% SDS solution, indicating a similar effect of the metal complexes and the detergent. The more gradual OD₂₆₀ increase in *E. coli* compared to *B. subtilis* was most likely caused by the less-permeable outer membrane characteristic of Gram-negative bacteria.³⁹ No enantiomeric difference was observed. As bacterial membranes have a higher proportion of negatively charged phospholipids compared with eukaryotic cells, as well as negatively charged teichoic acid and lipopolysaccharides,^{40–42} it is not surprising that the large binuclear ruthenium complex with a 4+ cationic charge would preferentially bind to the outer membrane of the bacterial cell.

Table 1 shows a summary of determined MIC and MBC values. The lowest MIC value was observed with Δ Ru-phen against *B. subtilis*, with an inhibitory effect comparable to that of tetracycline against the same bacteria. In contrast, Λ Ru-phen was less effective, with a 2-fold increase in its MIC value. The enantiomeric difference in Ru-phen was even higher when tested against *E. coli*, with Λ requiring an 4-fold higher concentration for the same bacteriostatic effect as Δ , but with an overall weaker inhibitory effect compared with *B. subtilis*.

In order to determine the MBC values for both ruthenium complexes a series of spot plating was performed (Figure 1

TABLE 1 MIC and MBC values^a

Compound	<i>B. subtilis</i>		<i>E. coli</i>	
	MIC	MBC	MIC	MBC
Δ	8	16	32	> 400
Λ	16	32	128	> 400
$\Delta\Delta$	ND ^b	150	ND ^b	300
$\Lambda\Lambda$	ND ^b	150	ND ^b	300
Tetracycline	8	16	< 1	< 1

^aMIC and MBC values indicated as μM . (Ru-phen: 1 μM = 0.82 $\mu\text{g/ml}$; biRu-phen: 1 μM = 1.63 $\mu\text{g/ml}$; Tetracycline hydrochloride: 1 μM = 0.48 $\mu\text{g/ml}$).

^bNot determined due to the ruthenium complex precipitating in the LB medium.

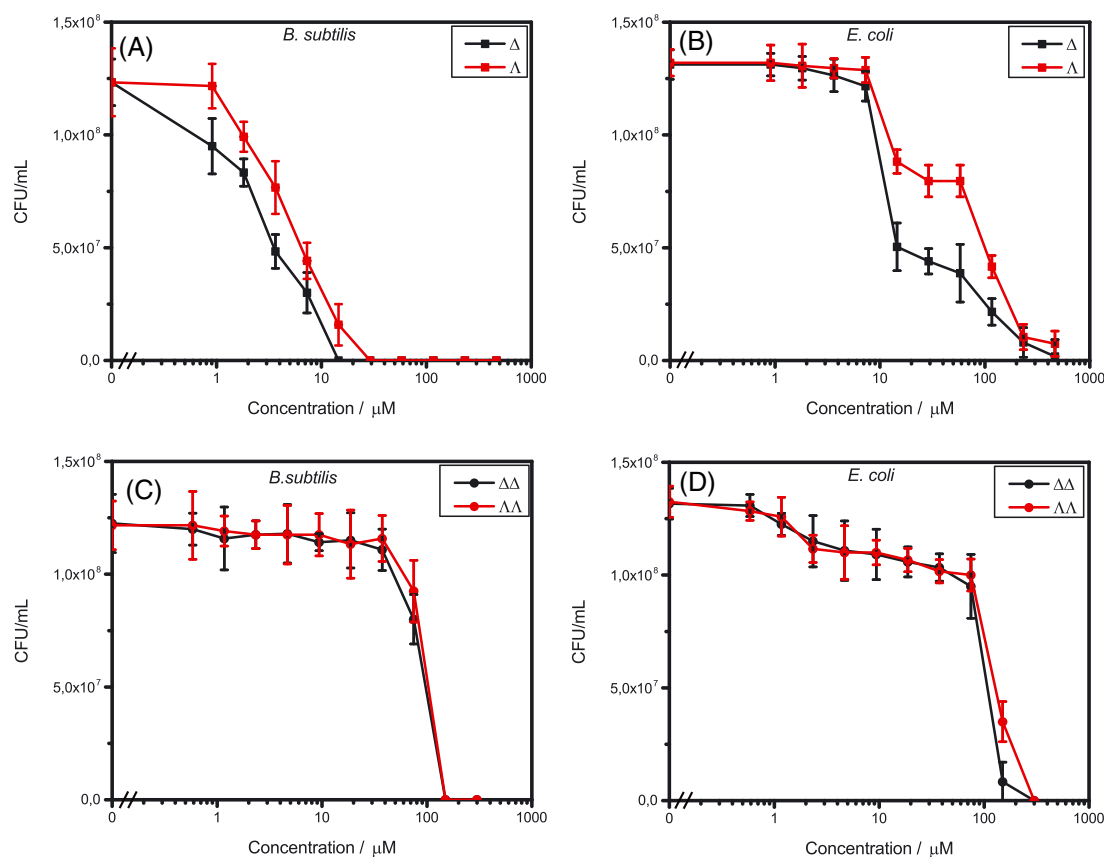


FIGURE 1 Surviving bacterial counts (CFU/mL) of *B. subtilis* (left column) and *E. coli* (right column) after 24-h exposure to Ru-phen (top row, a,b) and biRu-phen (bottom row, c,d) at 37°C. Each point is the mean of six replicate cultures. Error bars show standard deviations (SD: 3 σ); when not visible, these bars are as small as or smaller than the symbols plotted

[SD: 3σ] and Table 1). *E. coli* was never fully eradicated even with the highest concentrations tested. However, both Δ - and Λ Ru-phen showed a high bactericidal effect in *B. subtilis*, with a 2-fold higher efficiency in Δ compared to Λ . We also observed a plateau for the viability of *E. coli* at increasing concentrations of the mononuclear complexes (Figure 1b) before a sudden drop to almost zero bacterial growth. A similar yet much less pronounced effect can be seen for the binuclear complexes (Figure 1d). This response is likely the effect the emergence of a small subpopulation bacteria (so called “persisters”) that is able to survive antimicrobial exposure, and is part of a survival strategy already well established in *E. coli*.^{43–45} The slow-growing persister cells emerge as a response to environmental triggers such as antibiotics and acts as an insurance for the general bacterial population to survive in a stressful environment.

It is conceivable that DNA, being chiral due to its right-handed helical structure, would interact differently with the disymmetric nonintercalating peripheral pair of phen ligands on the ruthenium complex depending on chirality. Being either right- or left-handed, the Δ - and Λ -forms should fit differently into the grooves of the DNA. This could be observed in the early experiments with $[\text{Ru}(\text{phen})_3]^{2+}$ where the Δ - and Λ -forms differed in their individual binding modes with DNA^{17,46,47} and was further supported in studies with the intercalating analog Ru-phen, where Δ Ru-phen was determined to have a stronger binding affinity (2–5 times) for DNA compared to the Λ -form.^{48,49} The same chiral discrimination was observed in this study, with Δ Ru-phen showing both stronger bacteriostatic effect against both *B. subtilis* and *E. coli* compared to the Λ -form, indicating DNA binding as the primary antimicrobial mode of action. To test this hypothesis, emission of the bacterial cells was recorded after an overnight incubation with the ruthenium complexes. The bacterial cells were exposed to an excess of ruthenium complex, as indicated by the yellow color of the supernatants after washing. No emission of the supernatants was observed, thus suggesting that the cellular membranes of the bacteria had remained intact. Both Ru-phen and biRu-phen are known as “light-switch complexes,” meaning that they are completely quenched when unbound in aqueous solution, but show intense fluorescence when intercalated to DNA. Therefore, any fluorescence observed would suggest DNA binding. In both strains, the intensity of emission from the bacterial cells after incubation with Δ Ru-phen was significantly higher than that of the Λ -form (Figure 2). A slight redshift was observed for Λ Ru-phen in both strains, a phenomenon associated with DNA-complex interactions.^{35,50} The largest difference was observed in *E. coli*, consistent with the results from the metal susceptibility assays. The smaller difference in emission for cells of *B. subtilis* exposed to Δ Ru-phen or Λ Ru-phen could depend on the base composition of the genome. The genome of *B. subtilis* has a higher content of the A and T nucleic acids (56.5%)⁵¹ compared to the genome of *E. coli* (49.2%),⁵² and

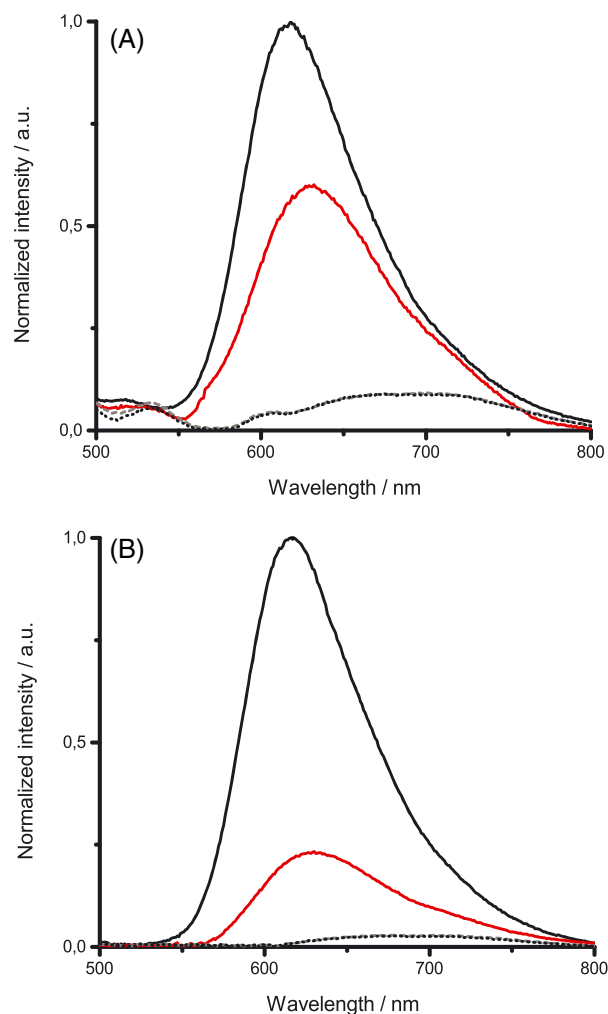


FIGURE 2 Normalized emission spectra of bacterial cells. (a) *B. subtilis*; (b) *E. coli* after overnight exposure to ruthenium complex (40 μM) at 37°C. The cells were washed twice and resuspended in 1 \times PBS solution. Emission from bacterial cells without exposure has been deducted. Black: Δ Ru-phen; red: Λ Ru-phen; black dot: $\Delta\Delta\text{biRu-phen}$; gray dash: $\Lambda\Lambda\text{biRu-phen}$

therefore could explain the less profound difference in intensities, in accordance with the early work by Hiort et al., where the same relative intensities were observed to differ less between Δ - and Λ - $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ when bound to AT-sequences compared to calf thymus DNA.³⁵ No emission in the binuclear complex was observed, indicating no interaction with DNA.

Figure 3 shows a confocal microscopic picture of Hoechst-stained *E. coli* that had been previously treated with either enantiomer of Ru-phen. The figure clearly shows emission from both enantiomers, but with much higher intensity from the Δ -form. In addition, the relation between the intensity of the Hoechst stain and the intensity of the complex appears to be inverse; the cells with the highest intensity of ruthenium complex appears to have very weak if any fluorescence from the Hoechst staining, and vice versa. This relation is apparent for both enantiomers. The ruthenium complex and the DNA-stain compete for binding in the minor groove of DNA, thus explaining why bacterial cells with high signal

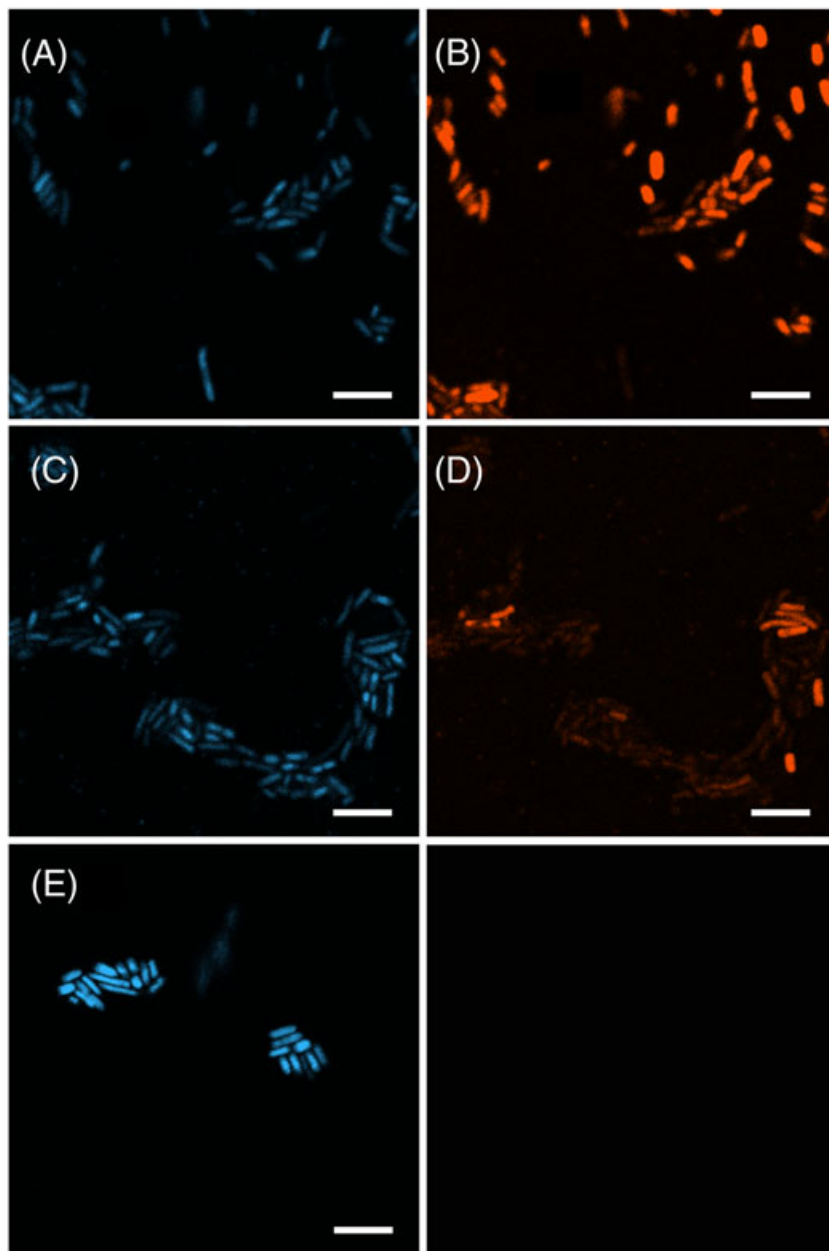


FIGURE 3 Confocal microscopy images of formaldehyde fixed *E. coli* samples incubated with Δ - (top row) and Λ Ru-phen (middle row) overnight and then stained with Hoechst 33342. (a) fluorescence, Hoechst 33342 (Δ -sample); (b) fluorescence, Δ Ru-phen; (c) fluorescence, Hoechst 33342 (Λ -sample); (d) fluorescence, Λ Ru-phen; (e) fluorescence, control sample *E. coli* stained with Hoechst 33342 (Bottom right: a black square for visual aid). Scale bar = 5 μ m

for Ru-phen have poor emission from the DNA-stain due to saturation on binding sites on the DNA. The same effect could be observed in *B. subtilis*, while the enantiomeric difference in emission was not as distinct (shown in Figure S3 in the Supporting Information). Furthermore, unexposed bacterial cells used as controls showed significantly stronger emission from the Hoechst dye compared to bacteria exposed to ruthenium complex, again indicating that ruthenium complex and the DNA binding dye competes for the same binding sites.

In conclusion, this is the first reported work of the potential usage of enantiopure $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ and $[\mu\text{-bidppz}(\text{phen})_4\text{Ru}_2]^{4+}$ as antimicrobial compounds. While the binuclear complex had no major toxic effect on bacterial cells, the mononuclear complex showed a high antimicrobial activity, especially in *B. subtilis*, where the effect was comparable to that of tetracycline (as given in Table 1). Importantly,

we observed the Δ -enantiomer of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ to be 2-fold more effective in both inhibiting and killing *B. subtilis* in comparison to the Λ -enantiomer. Our initial results suggest diastereomeric DNA interactions as the antimicrobial mode of action, which was further supported by stronger emission from Δ compared with Λ in bacterial cells exposed to the compounds. This study clearly demonstrates the potential of enantioselectivity in the bactericidal action of substitution inert transition metal complexes, and emphasizes the importance of studying enantiopure compounds.

4 | CONCLUSION

Two ruthenium(II) polypyridyl complexes were tested for their antimicrobial activity: $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ and $[\mu\text{-bidppz}(\text{phen})_4\text{Ru}_2]^{4+}$, both resolved into their pure

enantiomeric forms (Δ and Λ). Both enantiomers of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ displayed high bactericidal effect against Gram-positive *B. subtilis*, comparable to the antimicrobial activity of tetracycline, while having a more bacteriostatic effect on Gram-negative *E. coli*. In contrast, $[\mu\text{-bidppz}(\text{phen})_4\text{Ru}_2]^{4+}$ showed significantly lower antimicrobial activity against both bacterial strains with a bactericidal effect only at the highest concentrations tested.

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SUPPORTING INFORMATION

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