In vitro anticancer activity of binuclear Ru(II) complexes with Schiff bases derived from 5-substituted salicylaldehyde and 2-aminopyridine with notably low IC₅₀ values

Emira Kahrović, Adnan Zahirović, Sandra Kraljević Pavelić, Emir Turkušić & Anja Harej

To cite this article: Emira Kahrović, Adnan Zahirović, Sandra Kraljević Pavelić, Emir Turkušić & Anja Harej (2017): In vitro anticancer activity of binuclear Ru(II) complexes with Schiff bases derived from 5-substituted salicylaldehyde and 2-aminopyridine with notably low IC₅₀ values, Journal of Coordination Chemistry, DOI: 10.1080/00958972.2017.1308503

To link to this article: http://dx.doi.org/10.1080/00958972.2017.1308503
**In vitro** anticancer activity of binuclear Ru(II) complexes with Schiff bases derived from 5-substituted salicyaldehyde and 2-aminopyridine with notably low IC\textsubscript{50} values

Emira Kahrović\textsuperscript{a}, Adnan Zahirović\textsuperscript{a}, Sandra Kraljević Pavelić\textsuperscript{b}, Emir Turkušić\textsuperscript{a} and Anja Harej\textsuperscript{b}

\textsuperscript{a}Faculty of Science, Department of Chemistry, University of Sarajevo, Sarajevo, Bosnia and Herzegovina; \textsuperscript{b}Department of Biotechnology, Centre for High-Throughput Technologies, University of Rijeka, Rijeka, Croatia

**ABSTRACT**

The binuclear Ru(II) complexes with Schiff bases derived from 5-chlorosalicyaldehyde and 2-aminopyridine and its 5-substituted salicylideneimine homologues were tested **in vitro** against cervical carcinoma (HeLa), metastatic colorectal adenocarcinoma (SW620), lung adenocarcinoma (A549), breast adenocarcinoma (MCF-7), and human lung fibroblast (WI-38) cell lines. All compounds showed strong antiproliferative activity with extremely low IC\textsubscript{50} values. The compounds expressed strong activity against gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis*.

**1. Introduction**

The systematic study of metal-based drugs began with Rosenberg’s experiment on platinum-induced filamentous growth in *Escherichia coli* in the late sixties, resulting in the synthesis of numerous metal complexes with potential biological properties. In this class of compounds, platinum-based drugs cisplatin, oxaliplatin, and carboplatin still have a major importance in chemotherapy [1–3]. Significant side effects of platinum drugs and the need to improve selectivity of chemotherapeutics have introduced ruthenium compounds in the focus of interest over the last decades. It is thought that comparative advantage of ruthenium drugs primarily comes from its possibility to be transported by plasma protein transferrin and activated by reduction **in situ** [4, 5]. Interactions with different biomolecules including...
DNA, which would be responsible for anticancer activity of ruthenium compounds, are widely studied. To the present, the most promising ruthenium complexes are NAMI-A and KP1019 that showed superior activity toward lung metastases and colorectal carcinoma, respectively [6, 7].

Schiff bases are suitable chelating ligands for systematic study of metal complex compounds. There are some important advantages of Schiff bases derived from salicylaldehyde: (i) flexibility to coordinate metal ion as a neutral or anionic species through azomethine nitrogen, phenolic oxygen, also by additional atoms from aldehyde or amine part of molecule; (ii) with the number of O, N, and possibly additional donor atoms, it is possible to adjust redox potential and resistance toward hydrolysis as key properties for activation in biological systems. In low oxidation states, ruthenium demonstrates affinity toward N-, S-, and O-donor ligands. Ru(II) has significant affinity for N- and S-donor ligands, while Ru(III), as harder, prefers N- and O-donor ligands. Many ruthenium complexes with salicylideneimine are reported as very stable and, in most cases, inert compounds. Regarding the biological properties, ruthenium complexes with Schiff bases generally demonstrate significant antimicrobial activity compared to referent antibiotics [8–11], while anticancer activity is rather modest except in the case of biologically active co-ligands [12–15].

We recently published synthesis of a binuclear Ru(II) with Schiff base derived from 5-chlorosalicylaldehyde and 2-aminopyridine, hereinafter 1, which showed strong antimicrobial activity and moderate ability to bind calf-thymus (CT) DNA [16]. Inspired by the biological properties of the compound, we aimed to test its in vitro anticancer activity to prepare its homologues and compare their biological activity.

2. Materials and methods

2.1. Chemicals

All chemicals were analytical grade of purity and used without purification. Tetraethylammonium perchlorate was precipitated from aqueous solution of its bromide salt by addition of sodium perchlorate and was twice recrystallized from water and dried. Highly polymerized fibrous CT DNA Type I was obtained from Merck and purified using phenol-chloroform-isooamyl alcohol extraction until satisfactory $A_{260}/A_{280}$ ratio was reached and precipitated as sodium salt. Solutions of CT DNA (~5 mM) were prepared just before measurements by suspending solid nucleic acid in 0.1 M Tris-HCl buffer pH 7.42 and left overnight to assure hydration. Solutions were kept at 4 °C and used no longer than two days.

2.2. Methods

Elemental analysis was performed on a Perkin-Elmer 2400 Series CHNS/O Analyzer. Ruthenium content was determined from DMSO solution using graphite furnace atomic absorption spectroscopy according to published procedure [17].

Mass spectra were recorded in neutral reflector mode with firing rate 200 Hz, acquiring 1600 shots per spectrum in m/z range 10–1000 Da with 300 ns delay time using 4800 Plus MALDI TOF/TOF Analyzer, Applied Biosystems Inc. equipped with Nd:YAG laser, wavelength of 355 nm. Thiamine mononitrate and azithromycin were used as internal calibrants. $^1$H NMR spectra were measured with a 300 MHz Bruker BioSpin GmbH spectrometer at 293 K from dry d$_6$-acetone solutions of complexes. Chemical shifts are given in ppm relative to internal SiMe$_4$. 

E. KAHROVIĆ ET AL.
Infrared spectra were collected with a Perkin-Elmer BX FTIR spectrophotometer as KBr pellets from 4000 to 400 cm\(^{-1}\). Electronic spectra of complexes and ligands in CH\(_2\)Cl\(_2\) solutions were recorded using a Perkin-Elmer UV/Vis lambda 35 spectrophotometer from 200 to 700 nm.

Diamagnetic susceptibility was calculated based on measurements performed on a SQUID magnetometer MPMS-XL5 (Quantum Design) at 300 K. Cyclic voltammograms were recorded with Autolab potentiostat/galvanostat (PGSTAT 12) electrochemical workstation using three electrode system: glassy carbon electrode as working, Ag/AgCl as reference, and Pt wire as counter electrode with 0.1 V s\(^{-1}\) scan rate and step potential 0.025 V. Measurements were performed in dimethylformamide/sodium perchlorate and acetonitrile / tetraethylammonium perchlorate solutions. Conductivity measurements were carried out with millimolar complex solutions in dimethylformamide using a Phywe conductivity meter.

Interaction of complexes with CT DNA was investigated by electronic spectroscopy using spectrophotometric titration. Measurements were performed under physiological conditions (0.1 M Tris-HCl buffer, pH 7.42, 0.15 M NaCl) at ambient temperature with 5-min equilibration time. Spectra were collected from 200 to 700 nm after successive addition of CT DNA (~5 mM) into 2-mL solution of complex (~20 μM). CT DNA was compensated in the blank. Complexes were initially dissolved in dimethylsulfoxide (5 mM) and diluted with buffer to required concentration. Hydrolysis of complexes was investigated under same conditions in the absence of DNA.

Viscometric measurements were carried out using an Ubbelohde ASTM viscometer (0B size, \(C = 0.0057350\) cSt s\(^{-1}\)) at 25 ± 0.1 °C measuring time with digital stopwatch (±0.01 s). Viscosity of CT DNA (1.09 × 10\(^{-4}\) M) was measured in the presence of increasing concentrations of complexes (\(r_{\text{max}} = [\text{complex}]/[\text{DNA}] = 0.5\)) and correction was made for buffer viscosity in the presence of complex. In all measurements, 10 mM Tris-HCl buffer pH 7.42 was used.

The fluorescence spectra were recorded with a Perkin Elmer LS-55 Luminescence Spectrometer. Measurements were carried out by adding 1–4 up to final concentrations ranging from 0 to 7.40 × 10\(^{-6}\) M to CT DNA (2.20 × 10\(^{-5}\) M). Nucleic acid was previously treated with ethidium bromide (EB) (6.00 × 10\(^{-6}\) M) in 10 mM Tris-HCl buffer pH 7.42. All samples were excited at 510 nm and emission was recorded between 520 and 700 nm with emission maximum at 593 nm.

### 2.3. Bacterial cultures

Gram-positive bacteria methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), *Enterococcus faecalis* and gram-negative bacteria *Klebsiella pneumoniae* (wild type), *Klebsiella pneumoniae* (ESBL type), and *Pseudomonas aeruginosa* were collected from the Microbiology Laboratory of the Institute of Public Health of Canton Sarajevo.

### 2.4. Cell culturing

The cell lines HeLa (cervical carcinoma), SW620 (colorectal adenocarcinoma, metastatic), A549 (lung adenocarcinoma), MCF-7 (breast adenocarcinoma), and WI-38 (human lung fibroblast cell line) were cultured as monolayers and maintained in Dulbecco’s modified Eagle
medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 U mL\(^{-1}\) penicillin, and 100 μg mL\(^{-1}\) streptomycin in a humidified atmosphere with 5% CO\(_2\) at 37 °C.

### 2.5. Drug susceptibility testing

Disk-agar diffusion method was applied for antibacterial studies. Measurements were performed using bacterial suspensions of 0.5 McF turbidity. Volume of 50 μL of each complex (1.5 mg mL\(^{-1}\)) was inserted into drilled holes on Mueller-Hinton agar medium. Results are given as diameter (mm) zone of inhibition of bacterial growth after 24-h incubation at 37 °C. Vancomycin and gentamicin were used as reference antibiotics. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined by serial dilution technique counting the number of bacterial colonies after incubation. All measurements were conducted in triplicates.

The panel cell lines were inoculated onto a series of standard 96-well microtiter plates on day 0 at 5000 cells per well according to the doubling times of specific cell line. Test agents were then added in five, 10-fold dilutions (0.01 to 100 μM), and incubated for further 72 h. Working dilutions were freshly prepared on the day of testing in the growth medium. DMSO was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in the working concentrations (DMSO concentration never exceeded 0.1%). After 72 h of incubation, the cell growth rate was evaluated by performing the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay: experimentally determined absorbance values were transformed into a cell percentage growth (PG) using the formulas proposed by NIH and described previously [18]. This method directly relies on control cells behaving normally at the day of assay because it compares the growth of treated cells with the growth of untreated cells in control wells on the same plate; the results are, therefore, a percentile difference from the calculated expected value.

Anticancer activity of four Ru(II) complexes was expressed through IC\(_{50}\) values that were calculated from dose–response curves using linear regression analysis by fitting the mean test concentrations that give PG values above and below the reference value. Each test point was performed in quadruplicate in two individual experiments. The results were statistically analyzed (ANOVA, Turkey post hoc test at \(p < 0.05\)). Finally, the effects of the tested substances were evaluated by plotting the mean percentage growth for each cell type in comparison to control on dose–response graphs.

### 2.6. Synthesis

#### 2.6.1. Synthesis of Schiff bases

Schiff bases, \(N-(2\text{-pyridyl})-5\text{-X-salicylideneimine}\), where \(X = H, Br\) and NO\(_2\), were prepared via the reaction of equimolar amounts of 5-substituted salicylaldehyde and 2-aminopyridine in absolute ethanol at 70 °C [19].

#### 2.6.2. Synthesis of \([\text{Ru}_2\text{L}_2\text{Cl}_2(\text{Et}_2\text{NH})(\text{H}_2\text{O})]\cdot\text{nH}_2\text{O}\)

\([\text{Ru}_2\text{L}_2\text{Cl}_2(\text{Et}_2\text{NH})(\text{H}_2\text{O})]\cdot\text{H}_2\text{O}\), where \(L = N\text{-2-pyridyl-5-Cl-salicylideneimine}\), hereinafter 1, was synthesized according to published procedure and purity was checked by elemental analysis and IR spectrum [16]. The homologous complexes with Schiff bases
L = N-2-pyridyl-5-X-salicylideneimine, where X = H for 2, Br for 3 and NO₂ for 4, were synthesized according to the procedure mentioned above for chloro-derivative.

Ethanolic solution (5 mL) of RuCl₃·3H₂O (0.38 mmol, 100 mg) was added to mixture of appropriate Schiff base (0.76 mmol; 151 mg HL₂, 211 mg HL₃, 185 mg HL₄) and triethylamine (0.76 mmol, 0.10 mL) in ethanol (15 mL). The mixture was heated at 70 °C for 4 h after which the reduction to the half of initial volume was performed. Resulting solution was kept in ice-salt bath overnight; collected dark green solid was washed with cold water, ethanol and diethylether and dried at 60 °C. Recrystallization was performed from ethanol/dichloromethane, 1/1 v/v. Yields: 40–60%.

Compound 2. Dark green powder. Calcd for C₂₈H₃₃Cl₂ N₅O₄Ru₂ (%): C, 43.24; H, 4.28; N, 9.01; Ru, 26.23. Found (%): C, 43.18; H, 5.17; N, 9.64; Ru, 25.96. MALDI-TOF MS m/z: 664.8959.

IR (KBr), ν max (cm⁻¹): 1604(vs), ν sym(C=N); 1291(m), ν sym(C–O); 1022(m), δ bend(C₂N); 805(w), δ bend(Ru₂O); 470(w), δ in-plane(py). UV-vis [CH₂Cl₂], λ/ nm (log[ε/M⁻¹ cm⁻¹]): 230 (4.48), 255 (4.38), 295 (4.18), 427 (4.03), 688 (0.30). ¹H NMR (300 MHz, acetone-d₆): δ 10.04 s (2H(4)), 7.93 dd (2H(6), 3J = 6.12 Hz, 4J = 0.42 Hz), 7.78 d (2H(3), 3J = 1.68 Hz), 7.76 d (2H(8), 3J = 1.62 Hz), 7.59 td (2H(2), 3J = 8.25 Hz, 4J = 1.68 Hz), 7.07 t (2H(7), 3J = 4.71 Hz), 6.99 d (2H(5), 3J = 8.31 Hz), 6.93 d (2H(1), 3J = 7.71 Hz), 6.79 td (2H(12), 3J = 6.81 Hz, 4J = 0.68 Hz), 5.61 s (1H(9)), 3.44 q (4H(10), 3J = 6.84 Hz), 1.10 t (6H(11), 3J = 6.37 Hz).

Compound 3. Dark green powder. Calcd for C₂₈H₃₁Br₂Cl₂ N₅O₄Ru₂ (%): C, 36.02; H, 3.35; N, 7.51; Ru, 21.85. Found (%): C, 35.79; H, 2.47; N, 7.59; Ru, 21.65. MALDI-TOF MS m/z: 822.7169.

IR (KBr), ν max (cm⁻¹): 1604(vs), ν sym(C=N); 1288(m), ν sym(C–O); 1014(m), δ bend(C₂N); 808(w), δ bend(Ru₂O); 468(w), δ in-plane(py). UV-vis [CH₂Cl₂], λ/ nm (log[ε/M⁻¹ cm⁻¹]): 230 (4.36), 292 (4.27), 331 (4.23), 404 (4.05), 692 (0.31). ¹H NMR (300 MHz, acetone-d₆): δ 10.25 s (2H(4)), 8.75 s (2H(3)), 7.18–7.33 m (10H: 2H(1), 2H(2), 2H(5), 2H(6), 2H(7), 2H(8)), 5.61 s (1H(9)), 3.45 q (4H(10), 3J = 6.83 Hz), 1.10 t (6H(11), 3J = 6.38 Hz).

Compound 4. Dark green powder. Calcd for C₂₈H₃₃Cl₂ N₇O₉Ru₂ (%): C, 37.97; H, 3.75; N, 11.08; Ru, 23.03. Found (%): C, 37.12; H, 2.96; N, 11.13; Ru, 22.84. MALDI-TOF MS m/z: 754.8660.

IR (KBr) ν max (cm⁻¹): 1598(vs), ν sym(C=N); 1321(m), ν sym(C–O); 1019(m), δ bend(C₂N); 812(w), δ bend(Ru₂O); 468(w), δ in-plane(py). UV-vis [CH₂Cl₂], λ/ nm (log[ε/M⁻¹ cm⁻¹]): 231 (4.45), 299 (4.26), 335 (4.11), 416 (3.96), 695 (0.31). ¹H NMR (300 MHz, acetone-d₆): δ 10.05 s (2H(4)), 7.94 (2H(3)), 7.65 - 7.78 m (12H: 2H(1), 2H(2), 2H(5), 2H(6), 2H(7), 2H(8)), 5.61 s (1H(9)), 3.42 q (4H(10), 3J = 6.92 Hz), 1.11 t (6H(11), 3J = 6.37 Hz).

3. Results and discussion

3.1. Complex compounds

Since the binuclear Ru(II) complex containing Schiff base derived from 5-chlorosalicylaldehyde and 2-aminopyridine has previously showed strong antimicrobial activity against gram-positive bacteria and ability to bind DNA, the homologues were prepared and characterized, keeping in mind possible effect of substituents on biological activity. The proposed structure of compounds is presented in figure 1.

Two ruthenium atoms in (aqua)(dichloride)(diethylamine)bis[N-(2-pyridyl)-5-substituted-salicylideneimino)diruthenium(II,II) complexes are in a different octahedral coordination. Both ruthenium atoms are coordinated with one chloride, ONN donor atoms from the Schiff base, and bridging oxygen, while the sixth position is unequally occupied by one aqua ligand on one ruthenium atom and diethylamine ligand on the second metal atom. Complexes


2–4 were characterized on the basis of elemental analysis, different spectroscopic techniques, and magnetic measurements, confirming coordination environment and oxidation state of ruthenium atoms which corresponds to data published for 1. Compounds 2 and 4 were precipitated as dihydrate, whereas 3 contains one molecule of water.

3.2. Spectroscopic characterization

Mass spectra of the complexes showed isotopic distribution for ruthenium species at \(m/z\) 664.8959, 822.7169, and 754.8660, confirming molecular formulation for 2, 3, and 4, respectively.

\(^1\)H NMR spectra of all complexes confirmed the presence of deprotonated ONN anionic Schiff bases, azomethine groups, diethylamine, and aqua ligands. The lack of singlets at 10–12 ppm, which correspond to phenolic hydrogen, confirmed deprotonation of phenolic OH. The azomethine singlets appeared at \(\delta = 10.05\) ppm for 2 and 4 while 3, having bromide as a substituent, showed singlet at 10.25 ppm. The coordination of diethylamine to Ru(II) is proved on the basis of amine hydrogen with corresponding singlet at \(\delta = 5.61\) ppm in all compounds compared to 2 ppm in free diethylamine. Coordination of diethylamine to metal center resulted in a decrease of electron density around amine hydrogen moving the chemical shift to higher frequencies. Methylene and methyl groups are found in all spectra. Quartet of methylene hydrogens is located at \(\delta = 3.45–3.42\) ppm compared to 2.60 ppm in the free ligand. The coordination of diethylamine through nitrogen does not affect the position of methyl hydrogen triplet which appeared in all spectra at \(\delta = 1.11\) ppm. The multiplets in the 7.18–7.78 ppm region correspond to coupled hydrogen atoms of aromatic rings. Substituent on salicylaldehyde part of Schiff bases mainly influenced the chemical shift of hydrogen on adjacent carbon, \(H(3)\), which appears as doublet at 7.78 ppm for 2 and singlet at 8.75 and 7.94 ppm for 3 and 4, respectively. Proton NMR spectrum of 2 is shown in figure 2.

Infrared spectroscopy is a powerful technique for determination of ligand coordination mode. IR spectra of 2–4 confirm coordination of Schiff bases as tridentate anionic ligand. The typical shifts of azomethine, deprotonated phenol group, and pyridine in plane vibrations were observed by comparing the spectra of free and coordinated Schiff bases. Azomethine group was shifted by 5, 7, and 23 cm\(^{-1}\) for 2, 3, and 4, respectively, toward lower frequencies and appeared at 1604–1598 cm\(^{-1}\) after coordination. After coordination, asymmetric stretching of C–O was moved to higher frequencies (1288–1321 cm\(^{-1}\)) compared to free ligands (1278–1293 cm\(^{-1}\)) as a result C-O(Ru) bonding. The coordination through
pyridine nitrogen affected in plane vibrations of pyridine ring was moved to higher wavenumbers by about 17 cm\(^{-1}\) compared to free Schiff bases. The presence of diethylamine was confirmed by \(C_2\)N bendings which were shifted to lower frequencies in the range of 15–23 cm\(^{-1}\). The bridging role of phenolic oxygen between two Ru(II) atoms was confirmed by Ru,O bending absorptions which were found at 805–812 cm\(^{-1}\).

Electronic spectra of ruthenium compounds showed three ligand-centered transitions (Bands I–III) of coordinated Schiff bases which arise from intraligand \(\pi \rightarrow \pi^*\) and \(n \rightarrow \pi^*\) charge transfer. Band I was centered around 230 nm in all three complexes, whereas bands II and III were shifted to higher energy as a result of Schiff bases coordination through ONN-donor atoms. A new band assigned to MLCT (Ru(II) to ligand charge transfer) was found in the region 404–427 nm as further evidence of Schiff base coordination to Ru(II) atoms. The spin-allowed transition \(1A_{1g} \rightarrow 1T_{1g} (t_{2g}^6 \rightarrow \pi^*)\) of Ru(II) in strong ligand-field was found in all spectra in the region 688–695 nm.

Behavior of 1–4 in aqueous solution was investigated at physiological pH, as a precondition for the proper attribution of species liable for biological activity and spectral changes during the interaction with DNA. The title compounds showed significant resistance toward hydrolysis although the complexes contain chlorides which are typically readily leaving groups.

### 3.3. Magnetic susceptibility and electrochemical characterization

The magnetic susceptibility provides valid evidence for the electronic state of the central atom in the complexes or the degree of overlapping of atomic orbitals in the case of metal–metal interactions in polynuclear compounds. Experimental magnetic susceptibility \((\chi_\rho)\) at 300 K for ruthenium complexes 2–4 has negative values, confirming the presence of diamagnetic \(t_{2g}^6\) Ru(II) atoms (table 1).
Redox potential of a complex is certainly one of the most important characteristics for activity in biological systems. Potential drugs undergo different changes in biological systems including intra- and extracellular reduction or in situ reduction in the case of tumor cells. For 2–4, which contain Ru(II), this type of transformation is not expected; however, an electrochemical characterization of compounds contributes to understanding the effect of the substituent on the ligand on electronic density distribution in the molecule.

Cyclic voltammograms of 2–4 in dimethylformamide (DMF) and acetonitrile (MeCN) showed a quasi-reversible one-electron process assigned to Ru(III)/Ru(II) pair. Due to poor donor properties of MeCN, the cathode and anode peaks are more apparent and electron transfer more reversible with peak-to-peak separations $\Delta E = 0.175$ to $0.326$ V, compared to behavior in DMF. The electron transfer in DMF is slightly more controlled by diffusion, resulting in an increased peak separation with $\Delta E = 0.551–0.726$ V. All three compounds showed half-wave potentials $E_{1/2}$ in the range from -0.850 to -0.862 V in MeCN and -0.537 to -0.600 V in DMF. Negative values are a result of bonding hard oxygen to Ru(II), which is considered to be rather soft in character (table 1). The substituent on the aldehyde ring of Schiff bases, bearing also phenolic oxygen, affects $E_{1/2}$ which increases in the order Br < NO2 < H (−0.600, −0.587 and -0.537 V, respectively). Although bromo- and nitro-substituents have electron-withdrawing properties (inductive effect), stronger resonance effect of lone pair delocalization through aromatic ring is more dominant thus increasing electron density of molecule and rendering Ru(II) oxidation compared to H-homologue.

The non-electrolytic nature of 2–4 (0.1 mM) is in accord with conductivity in DMF (12.1–23.0 $\mu$S cm$^{-1}$) compared to reference 0.1 mM NaClO$_4$ (220 $\mu$S cm$^{-1}$).

### Table 1. Electrochemical, magnetic susceptibility, and conductivity data for 2–4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cyclic voltammetry, DMF (MeCN)</th>
<th>$\chi_\rho \text{ (cm}^3 \text{ g}^{-1})$</th>
<th>$S$ (\muS cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$E_c$ (-1.025)</td>
<td>-0.900 (−1.025)</td>
<td>-0.174 (−0.699)</td>
</tr>
<tr>
<td>3</td>
<td>$E_a$ (-0.950)</td>
<td>-0.875 (−0.950)</td>
<td>-0.324 (−0.775)</td>
</tr>
<tr>
<td>4</td>
<td>$E_{1/2}$ (-0.850)</td>
<td>-0.900 (−0.950)</td>
<td>-0.274 (−0.750)</td>
</tr>
</tbody>
</table>

### 3.4. Biological properties

#### 3.4.1. Interaction with CT DNA

The study of the interaction with DNA is usually one of the first steps in an evaluation of possible biological properties of a potential drug. DNA is a crucial molecule for the most important processes which take place in living systems. Although there is no exact evidence whether unique molecule or process is responsible for biological activity of ruthenium complexes, especially anticancer activity, indisputably DNA might be assumed as one of the primary targets. Small molecules can be bound by covalent mode to DNA like in the case of cisplatin which is activated by hydrolysis. Metal complexes with aromatic ligands rather bind DNA as groove binders or by intercalation. In any case, the binding of metal complex to DNA can suppress replication of nucleic acid. We recently reported that 1 showed moderate ability to bind DNA. The ability of its homologues 2–4 toward CT DNA was investigated by spectroscopic titration and the binding constants were calculated based on the equation:

\[
\frac{[\text{DNA}]}{\varepsilon_a - \varepsilon_f} = \frac{[\text{DNA}]}{\varepsilon_b - \varepsilon_f} + \frac{1}{K_b (\varepsilon_b - \varepsilon_f)} \tag{1}
\]
where \( \varepsilon_a, \varepsilon_f, \) and \( \varepsilon_b \) are extinction coefficients of complex in apparent, free, and bound forms, respectively. Binding constants \( (K_b) \) were calculated as intercept/slope ratio by plotting \([\text{DNA}] \) versus \([\text{DNA}] / (\varepsilon_f - \varepsilon_a) \) (figure 3 and figure S7).

Moderate \( K_b \) values and their variations for different substituents imply non-covalent hydrophobic binding to nucleic acid. Compound 2 with \( X = \text{H} \) showed the highest affinity to bind CT DNA, whereas Br or NO\(_2\) decreases the binding constants by reducing the hydrophobicity of the entire molecule.

Intrinsic binding constants \( (K_b) \) for 1–4 have values ranging from 1.14 to \( 4.01 \times 10^4 \text{ M}^{-1} \), which are significantly lower than those for classical intercalators such as ethidium bromide (7 \( \times \) \( 10^7 \text{ M}^{-1} \)) [20] and \([\text{Ru} (\text{imp})_2(\text{dppz})]^2^+ \) (2.19 \( \times \) \( 10^7 \text{ M}^{-1} \)) [21] or partial intercalators such as \([\text{Co} (\text{phen})_2(\text{dppz})]^3^+ \) (9.09 \( \times \) \( 10^5 \text{ M}^{-1} \)) [22] and \([\text{Ru} (\text{bpy})_2(\text{HPIP})] ([\text{PF}_6])_2 \) (6.5 \( \times \) \( 10^5 \text{ M}^{-1} \)) [21] but are comparable to those of groove binders such as \([\text{Ru} (\text{phen})_3]^2^+ \) (3 \( \times \) \( 10^4 \text{ M}^{-1} \)) [23] or \([\text{Cu}(5-\text{OMe-sal-CA})_2] \) (1.55 \( \times \) \( 10^4 \text{ M}^{-1} \)) [24]. Moreover, salicylideneimine complexes in absence of \( \pi \)-extended rings are reported to have \( K_b \) values \( 10^4 \text{ M}^{-1} \) [25–28]. Moderate hypochromism along with no MLCT band shifting and \( K_b \) values of \( 10^4 \text{ M}^{-1} \) order suggest that 1–4 bind DNA primarily through groove.

In order to prove binding mode, we investigated the effect of complexes on DNA viscosity. In the absence of structural data on interaction of metal complex with DNA hydrodynamic measurements, as very sensitive to change in length of DNA, are the most critical tests for determination of a binding mode. Effect of increasing concentrations of 1–4 to third root of relative viscosity of CT DNA is shown in figure 4.

Intercalating molecules significantly increase viscosity of double helix DNA. On the other hand, electrostatic binding of small molecules results in decrease of DNA viscosity. Groove-binders can cause both increase and decrease of viscosity, but generally the changes are not significant [29, 30]. Complexes 1–4 induce only moderate increase (7–15%) of third root of relative DNA-viscosity at relatively high \([\text{complex}] / [\text{DNA}] \) ratios \( (r_{\text{max}} = 0.50) \), thus, supporting groove binding.

Furthermore, fluorescence spectroscopic studies (figure 5) were carried out to confirm the binding mode of 1–4 to CT DNA. Studies were done using CT DNA previously treated

**Figure 3.** Spectroscopic titration of 2 (1.77 \( \times \) \( 10^{-5} \text{ M} \)) with CT DNA (4.90 \( \times \) \( 10^{-3} \text{ M} \), \( A_{260}/A_{280} = 1.81 \)) in 0.1 M Tris-HCl buffer pH 7.42, 150 mM NaCl, \( T = 295 \text{ K} \), \( t = 5 \text{ min} \).
with ethidium bromide (EB) that shows emission when intercalating between adjacent DNA base pairs. The presence of a complex that shows affinity toward DNA causes quenching of DNA-EB adduct emission by replacing EB and/or by accepting the excited state electron of EB through a photoelectron transfer mechanism [31]. The presence of 1–4 in increasing concentrations (0–7.40 × 10⁻⁶ M) caused minor reduction of emission intensity of intercalating EB, suggesting that complexes bind to DNA.

The fluorescence quenching of EB bound to DNA by the complexes is in agreement with the classic linear Stern–Volmer equation:
where $I_0$ and $I$ are the emission intensities in the absence and presence of the quencher, respectively, $K_{SV}$ is the Stern–Volmer quenching constant, and [complex] is total concentration of the complex-quencher (figure S9) [32]. The $K_{SV}$ values of $7.85 \times 10^3–1.39 \times 10^4$ M$^{-1}$ for the complexes suggest moderately strong interaction with DNA.

The apparent binding constant $(K_{app})$ was also calculated from the equation:

$$K_{app} = K[EB]$$

where $K$ is binding constant of EB to DNA $(1 \times 10^7$ M$^{-1}$), [EB] is concentration of EB $(6.00 \times 10^{-6}$ M), and [complex] is the concentration of complex that causes 50% quenching of the initial EB fluorescence [32]. The $K_{app}$ values $4.71–8.33 \times 10^5$ M$^{-1}$ suggest moderately strong competition of complexes with EB for its binding sites, but are still too low to suggest classical intercalation.

Results of interaction of 1–4 with CT DNA are summarized in table 2. All measurements suggested that reactivity to CT DNA follows the order $2 > 1 > 4 > 3$.

### 3.4.2. In vitro anticancer activity

Among platinum metals that are currently being studied for their antitumor activity ruthenium occupies a major place. The title compounds, binuclear Ru(II) complexes with Schiff bases derived from 5-substituted salicylaldehyde and 2-aminopyridine were tested in vitro against cancers, cervical carcinoma (HeLa), metastatic colorectal adenocarcinoma (SW620), lung adenocarcinoma (A549), breast adenocarcinoma (MCF-7), and human lung fibroblast (WI-38), as control cell line in order to find potential candidates for further evaluation. Tested compounds exerted strong antiproliferative effects in the very low micromolar range and were active in the range of 0.1–100 μM (table 3).

To the present, two different concepts are in the focus of global efforts to find adequate response in the fight against cancer. Classical concept of chemotherapy is associated with ability of drugs to bind DNA as a primary target, thus, resulting in the blockage of replication and transcription. Such drugs, i.e. platinum-based drugs, are non-selective and show serious general toxicity [33, 34]. Current concept in the development of new selective chemotherapeutic agents is addressed to targeted therapy that implies the compounds are able to disrupt specific cellular signaling pathways which are responsible for many processes, especially the formation of metastases with very poor response to chemotherapeutic drugs. Recent study showed capability of NAMI-A to scavenge NO, thus, suppressing angiogenesis processes that are believed to be responsible for solid tumors growth and, especially, their development into metastasis [35]. The classic concept requires drugs with low IC$_{50}$ values.

### Table 2. Interaction of 1–4 with CT DNA.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spectroscopic titration</th>
<th>Viscosimetry</th>
<th>Fluorescence studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda$ (nm)</td>
<td>$K_b$ (M$^{-1}$)</td>
<td>$(\eta/\eta_0)^{1/3}$</td>
</tr>
<tr>
<td>1</td>
<td>235</td>
<td>3.82 $\times$ 10$^4$ [16]</td>
<td>1.13</td>
</tr>
<tr>
<td>2</td>
<td>241</td>
<td>4.01($\pm$0.09) $\times$ 10$^4$</td>
<td>1.15</td>
</tr>
<tr>
<td>3</td>
<td>236</td>
<td>1.14($\pm$0.11) $\times$ 10$^4$</td>
<td>1.07</td>
</tr>
<tr>
<td>4</td>
<td>235</td>
<td>1.48($\pm$0.12) $\times$ 10$^4$</td>
<td>1.12</td>
</tr>
</tbody>
</table>

$r = [\text{complex}]/[\text{DNA}] = 0.50.$

$$\frac{I_0}{I} = 1 + K_{SV}[\text{complex}]$$

$$K_{app}[\text{complex}] = K[EB]$$
as in the case of Pt drugs. On the other hand, NAMI-A, which is selective toward lung metastasis, does not exhibit significant toxicity in vitro. In any case, in light of both concepts, low IC$_{50}$ values are encouraging for further testing of potential drugs.

All Ru(II) compounds 1–4 showed very low IC$_{50}$ values, few-fold lower than cisplatin. Compound 2 showed strong antiproliferative activity with extremely low IC$_{50} = 0.68$ μM against lung adenocarcinoma (A549). Anticancer activity corresponds to the order 2 > 1 ~ 3 > 4, indicating an effect of substituent on aldehyde ring of Schiff base. At the same time, 1–3 showed strong activity against control cell line, whereas 4 containing NO$_2$-substituent on Schiff base shows somewhat different feature. IC$_{50}$ values of 4 for SW620, MCF-7, and HeLa were few-fold lower than for a control cell line that could be a promising result for further study of the compounds. Based on only moderate activity of Ru(II) compounds toward CT DNA, as a primary target for Pt drugs, and very strong in vitro anticancer activity against several cell lines, it seems that 1–4 work via different mechanisms than cisplatin.

Numerous Ru(II) and Ru(III) complexes were tested in vitro against HeLa, MCF-7, A549, and SW620 cell lines, however among the hundreds of complexes, only a very limited number showed low IC$_{50}$ values. HeLa is the most tested cancer cell line. Cytotoxicity of ruthenium complexes against this cancer cell line varies from negligible, e.g. in the case of some Ru(II) substituted-dipyridyl ligands bearing ammonium group (IC$_{50} > 400$ μM) [36], up to a very high activity. To our knowledge, polypyridyl Ru(II) with anthraquinone are the compounds with the strongest activity against cervical carcinoma cells with IC$_{50}$ ranging from 0.5 to 17 μM in both, normoxic and hypoxic conditions [37]. The same compounds exhibit similar cytotoxicity against A549 cell line with IC$_{50}$ from 0.5 to 1.9 μM. Disadvantage of the compounds is also high cytotoxicity against control cell lines (4.5–5 μM). Relatively high cytotoxicity against lung adenocarcinoma was shown by Ru(II)-arene complex coordinated with phenanthroimidazole with IC$_{50}$ about 16 μM [38]. The same compound showed insignificant activity against SW620 cell line with IC$_{50} > 100$ μM. Ru(II) complexes with hydrazone type of ligands showed high cytotoxicity against MCF-7 with IC$_{50}$ from 2 to 6 μM [39]. Regarding the ruthenium Schiff bases, poor cytotoxic effect against MCF-7 (IC$_{50} = 90$ to > 100 μM) was reported for Ru(III) with tetradeutate ligands [40]. Very strong antiproliferative activity against human breast adenocarcinoma cell line was shown by a series of Ru(II) complexes with tridentate pyridine Schiff-base ligand and bidentate co-ligands (IC$_{50} = 0.8–4.2$ μM) [41]. Compared to the ruthenium complexes mentioned above, which express the strongest anticancer activity, 1–4 demonstrated similar or even better antiproliferative activity, especially in the case of metastatic colorectal adenocarcinoma (SW620).

### Table 3. IC$_{50}$ values (μM) for 1–4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>SW620</th>
<th>A549</th>
<th>MCF-7</th>
<th>HeLa</th>
<th>WI38</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.26 ± 0.17</td>
<td>1.05 ± 0.79</td>
<td>4.59 ± 0.42</td>
<td>2.23 ± 0.82</td>
<td>2.74 ± 0.48</td>
</tr>
<tr>
<td>2</td>
<td>1.99 ± 0.56</td>
<td>0.68 ± 0.88</td>
<td>4.09 ± 0.78</td>
<td>1.66 ± 0.48</td>
<td>2.51 ± 98.88</td>
</tr>
<tr>
<td>3</td>
<td>3.00 ± 0.20</td>
<td>1.38 ± 0.41</td>
<td>4.70 ± 0.16</td>
<td>2.26 ± 1.09</td>
<td>4.01 ± 2.08</td>
</tr>
<tr>
<td>4</td>
<td>5.24 ± 0.86</td>
<td>26.85 ± 24.9</td>
<td>8.13 ± 0.95</td>
<td>5.80 ± 4.34</td>
<td>31.62 ± 69.51</td>
</tr>
</tbody>
</table>

3.4.3. **Antimicrobial activity**

An intensive look for new compounds with antimicrobial activity originates from the growing abilities of bacterial strains to develop resistance to the drugs in use. Special attention is being paid to *Enterococci* and *Staphylococci* which are able to quickly adapt to antibiotics in
use. Numerous organic molecules show positive antimicrobial activity, but their metal complexes have improved effect probably due the structural order in complex which is important for biochemical reactions. Schiff bases that are widely used as ligands i.e. salicylideneimines have been reported for their antimicrobial activity, generally against gram-positive pathogens, including *E. coli* and *S. aureus* [42–44]. Antimicrobial activity *in vitro* for 2-4 was tested by disk diffusion method and by determining MIC and MBC values against methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus* and *Enterococcus faecalis* (table 4). We already published strong antimicrobial activity for 1. The complexes showed no significant activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains.

*In vitro* antimicrobial screening showed that 2 and 4 have good capability to inhibit *Staphylococcus aureus* growth (zone inhibition 18–21 mm) compared to vancomycin as a reference antibiotic (28 mm). Both compounds showed strong bactericidal activity against MRSA and MSSA. The inhibitory effect of 2 and 4 was found to be bactericidal, meaning that agents cause the death of pathogens (MBC/MIC ratio = 1–2) [45]. Bromo-derivative 3 showed significantly weaker activity against *S. aureus* strains, although it has the same MIC and MBC value as 2 for *Enterococcus faecalis*. Nitro-derivative 4 showed excellent activity against *Enterococcus faecalis* with promisingly low MIC and MBC values (1.46 and 0.73 μg mL⁻¹) that are very close to vancomycin (0.73 and 0.73 μg mL⁻¹).

### 4. Conclusion

Binuclear Ru(II) complexes with Schiff bases derived from 5-substituted salicylaldehyde and 2-aminopyridine, formulated as [Ru₂L₂Cl₂(H₂N)(H₂O)]·nH₂O, where L = N-2-pyridyl-5-X-salicylideneimine (X = H, Cl, Br and NO₂), showed strong *in vitro* anticancer and antimicrobial activity, however moderate ability to bind CT DNA. IC₅₀ values for H, Cl, and Br derivatives are in the range of very low micromolar concentrations, varying from 0.68 μM against adenocarcinoma (A549) cells to 4.70 μM for breast adenocarcinoma (MCF-7). All compounds exerted very significant activity against metastatic colorectal adenocarcinoma (SW620) with low IC₅₀ values (1.99–5.24 μM), although only NO₂ derivative showed lower toxicity to WI38 control cell line (31.62 μM) thereby making this compound a potential candidate for further development. These complexes showed strong activity against gram-positive bacterial strains *S. Aureus* and *E. faecalis*, especially nitro-derivative with bactericidal activity toward MRSA that is very close to vancomycin. Although there are not many reasons to discuss a structure–activity relationship in this short series of Ru(II) binuclear complexes, since composition difference comes only from 5-X-substituent on aldehyde ring from Schiff base, a different activity of nitro derivative toward cancer and control cell lines suggests further research.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MSSA</th>
<th>MRSA</th>
<th>Enterococcus faecalis</th>
<th>MSSA</th>
<th>MRSA</th>
<th>Enterococcus faecalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20</td>
<td>21</td>
<td>17</td>
<td>5.86 (2.92)</td>
<td>2.92 (2.92)</td>
<td>11.70 (11.70)</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>13</td>
<td>16</td>
<td>46.87 (46.87)</td>
<td>46.87 (46.87)</td>
<td>11.70 (11.70)</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>21</td>
<td>25</td>
<td>5.86 (2.92)</td>
<td>2.92 (2.92)</td>
<td>1.46 (0.73)</td>
</tr>
<tr>
<td>vancomycin</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>0.73 (0.73)</td>
<td>0.73 (0.73)</td>
<td>0.73 (0.73)</td>
</tr>
</tbody>
</table>
Acknowledgement

We greatly appreciate access to equipment in possession of University of Rijeka within the project “Research Infrastructure for Campus-based Laboratories at University of Rijeka”, co-financed by European Regional Development Fund (ERDF).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by Federal Ministry of Education and Science, Bosnia and Herzegovina [grant number 05–39-3936–1/15] through authors from Bosnia and Herzegovina.

References