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CT DNA, BSA and Antiproliferative Activity of Ru(II) Bipyridine Complexes Containing Schiff Bases Derived from Amino Acids

Irnesa Osmanković,[1] Emir Turkušić,[1] Adnan Zahirović,[1] Marijeta Kralj,[2] Lidija Uzelac,[2] Emira Kahrović[1,*]

[1] Laboratory for Inorganic and Bioinorganic Chemistry, Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
[2] Ruđer Bošković Institute, Division of Molecular Medicine, Bijenička c. 54, 10000 Zagreb, Croatia
* Corresponding author's e-mail address: emira_kahrovic@yahoo.com

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Abstract: Complexes of general formula $[Ru(bpy)_2(L)]CF_3SO_3$, where bpy = 2,2′-bipyridine, and L = Schiff bases derived from salicylaldehyde and amino acids (glycine (**1a**), cysteine (**1b**), methionine (**1c**) and phenylalanine (**1d**)) were synthesized. Characterization based on elemental analysis, Ru content, mass, infrared and electronic spectra confirmed RuN_5O coordination unlike **1b** where coordination occurred *via* azomethine nitrogen and cysteine sulfur. Cyclic voltammograms, except **1b**, showed several quasi-reversible redox pairs in the positive potential range, the first located at about 0.5 V, corresponding to similar heteroleptic Ru(II) bipyridyl complexes. Biological activity was tested by interactions with DNA and BSA. DNA binding constants of order 10^3 M^{-1}, suggest groove binding due to bpy ligand and hydrogen bonding of the OH and CO groups from the imine moiety. *In vitro* BSA protein inhibition assay performed by spectrofluorimetry showed Complex : BSA binding in 1 : 1 ratio with K_b of 10^4 M^{-1} order. Cytotoxicity studies by MTT assay for 72 h of drug action revealed activity of **1a** and **1d** against breast cancer MCF-7 cells with IC_{50} values 32 ± 8 and 26 ± 1μM, respectively.

Keywords: ruthenium, polypyridyl, Schiff bases, amino acids, biomolecules, cytotoxicity.

INTRODUCTION

In recent decades, ruthenium complexes have attracted an extended attention, especially due to their antitumor activities. Following the long-standing interest in cisplatin and similar compounds, the introduction of ruthenium into the focus of interest has resulted in hundreds of new compounds. Almost the major attention was focused on water soluble ruthenium dimethyl sulfoxide compounds,[1] Clarke and Keppler classes.[2,3] The main target of ruthenium antitumor compounds is DNA,[4] due to its unique function in cell replication. In the context, anticancer activity includes cell accumulation, activation, adduct formation with DNA, which can result in cellular apoptosis. Depending on the structure of Ru compounds, different modes of transport and activation in biological system are possible. Unlike cisplatin, which enters the cell by passive diffusion, some ruthenium compounds, such as NAMI-A, are thought to be transported by transferrin, thus reducing side effects compared to cisplatin.[5] Ruthenium is thought to have certain advantages over Pt drugs because of the ability to mimic the iron properties when bound to transferrin and other biomolecules. Due to significantly higher oxygen consumption, cancer cells have a specially developed transferrin receptor system that can be used to transport Ru compounds to target cancer cells.[6] Ruthenium complexes can be activated by hydrolysis, which implies the presence of easily leaving ligand, most often chloride, which ultimately opens a coordination position for binding of the target biomolecule, similar to the activation of cisplatin. The second model involves activation by "reduction *in situ*", due to hypoxicity (reduction ability) of solid cancer cells. Some anticancer ruthenium complexes have been described as light-activated compounds. The cytotoxic effects of such ruthenium polypyridyl photosensitizer complexes have been described to be based on the production of singlet oxygen ($^1O_2$) and other reactive oxygen species (ROS), with the formation of reactive intermediates that

form covalent bonds with biological molecules.[7] In the last 20 years, research on polypyridyl ruthenium complexes has grown exponentially, emphasizing their outstanding physicochemical relevance and biological activity starting from the basic compound $Ru(bpy)_3^{2+}$ to numerous derived compounds that are being investigated for various applicability, as potential drugs, but also for their photochemical and redox properties.[8,9] Hundreds of compounds including especially interesting complex-containing bioconjugates, which means a strategy of covalent binding to polypyridyl of one biomolecule or bioactive compound as a fragment, are being investigated.[10,11] Various biomolecules such as peptides, amino acids, proteins etc. are being used for the functionalization of polypyridyl molecules in metal complexes.[12] Numerous previously reported polypyridyle Ru-complexes with different co-ligands have been reported to have promising biological potential, particularly in the light of anticancer activity. In addition to the structural aspect of the compound and IC_{50} values, a number of studies emphasize the mechanism of biological action, delivery and activation. Many ruthenium polypyridyl complexes showed ability to enter the cytoplasm and accumulate in cell nuclei, causing apoptosis.[13,14] Another interesting biological aspect of some ruthenium bis(bipyridine) complexes with serotonin and γ-aminobutyric acid ligands, known as cage compounds, are described as powerful tools for rapid and localized delivery of bioactive neurotransmitter substances**.** Unlike many studies of anticancer properties and cellular localization of numerous ruthenium complexes that have been investigated in recent decades, significant interest in their antimicrobial properties has recently been devoted.[15–18] A number of ruthenium polypyridyl heteroleptic complexes with biomolecules as co–ligands have been designed thus tuning relevant properties to improve biological activity. Since several ruthenium complexes having Schiff bases have showed strong biological activity[19,20] we were motivated to prepare and report the chemistry of four novel mixed-ligand polypyridyl complexes of ruthenium(II) using 2,2'-bipyridine and salicylaldimine Schiff bases derived from amino acids glycine, cysteine, methionine and phenylalanine as co-ligands and test their ability to bind DNA and BSA. Previous studies of metal–amino acid Schiff base complexes were mostly focused on the complexes with divalent Fe, Ni, Cu, Zn, and trivalent Mn,[21–26] while the ruthenium chemistry having Schiff bases derived from amino acids appears to remain much less explored.

EXPERIMENTAL

Materials

Most of the chemicals were commercially obtained and used without further purification. The 2,2'-bipyridine (bpy) was recrystallized from hot *n*-hexane. *Cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$ was prepared by refluxing a dimethylformamide mixture of $RuCl_3$ and 2,2'-bipyridine in the presence of excess LiCl, precipitated with acetone and recrystallized from aqueous LiCl solution under nitrogen.[27] Reagent grade glycine (Gly), L-cysteine (Cys), L-methionine (Met) and L-phenylalanine (Phe) were purchased from Merck. Calf-thymus DNA was supplied by Sigma, Type I, fibers (A_{260} / A_{280} > 1.8). Bovine serum albumin (BSA, ≥ 98 %) was obtained from Sigma as a lyophilized powder.

Physical Measurements

Elemental analyses of C, H, N were performed using a Perkin Elmer 2400 Series CHNS/O analyzer. Ruthenium content was determined spectrophotometrically using 1,10-phenanthroline.[28] Mass spectra were recorded using nano-liquid chromatography and high resolution mass spectrometry (nanoUPLC-ESI-qTOF) coupled techniques on a nanoAcquity Ultra Performance LC and Synapt G2–Si in the positive ion reflector mode using leucine enkepahlin matrix. Infrared spectra were collected as KBr pellets in the 4000–400 cm^{-1} region with a Perkin Elmer BX FTIR. Absorption spectra were acquired in the 200–700 nm range in methanol solution using Perkin Elmer Lambda 35. Electrochemical measurements were done using a three-electrode system with a Pt wire as working, a Pt wire as a counter and Ag/AgCl as a reference electrode *via* a salt bridge in acetonitrile solution and 0.1 M tetrabutylammonium hexafluorophosphate as supporting electrolyte using a BioLogic SP–300 potentiostat / galvanostat electrochemical workstation in the potential range of −0.1 to 1.1V, with scan rate 100mV s^{-1}. Hydrolitic profiles at physiological pH 7.42 in 10 mM Tris-HCl buffer were investigated spectrophotometrically. Concentrated stock solutions of the complexes were prepared by initial dissolution of the compounds in a small amount of methanol and diluting to the required concentrations. Electronic spectra of the complexes ($2.5 \cdot 10^{-5}$M) were collected in the 200–700 nm region every 10 min during 2 h and after 24 h.

Stock solution of CT DNA (A_{260} / A_{280} = 1.86) was prepared prior to measurements by suspending the solid nucleic acid in 10 mM Tris-HCl (tris(hydroxymethyl)aminomethane) buffer pH 7.42, and left for good hydration overnight. The concentration was determined based upon the extinction coefficient 6600 M^{-1} cm^{-1} at 260 nm. The interaction of complexes with CT DNA was done at physiological pH 7.42 in 10 mM Tris-HCl buffer. Concentrated stock solutions of complexes were prepared in methanol and then diluted to the required concentrations. Spectrophotometric titrations of complexes ($5 \cdot 10^{-5}$M) with CT DNA ($7 \cdot 10^{-3}$ M) were carried out by adding µL amounts of CT DNA (0–80 µL) to the solutions of complex compounds (2 mL) and acquiring spectra in 200–700 nm region with a 2 min equilibrium time.

Interaction of complexes with BSA was carried out in 10 mM Tris-HCl buffer solution at pH 7.42, based on spectroscopic titration method. The stock solution of protein (1 μM) was prepared prior to the measurements. BSA concentration was determined spectrophotometrically using extinction coefficient of 43 824 M^{-1} cm^{-1}. Stock solutions of complexes (0.1 mM) were prepared in methanol. Titration experiments were carried out at room temperature by adding the microliter amounts (10–80 μL) of a stock solution of the complex to the protein solution (2.0 mL) with an equilibration time of 2 minutes. Emission spectra were recorded in 290–420 nm range with excitation wavelength at 278 nm.

In vitro Antiproliferative Activity

The cell lines H460 (lung carcinoma, large cell lung cancer), MCF-7 (breast adenocarcinoma) and SW 620 (colorectal carcinoma) were cultured as monolayers and maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), 2mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a humidified atmosphere with 5 % CO_2 at 37 °C.The panel cell lines were inoculated onto a series of standard 96-well microtiter plates on day 0, at $1{\cdot}10^4$ to $3{\cdot}10^4$ cells mL^{-1}, depending on the doubling times of specific cell line. Test agents were then added in five 10-fold dilutions (10^{-8} to 10^{-4} M) and incubated for a further 72 h. Working dilutions were freshly prepared on the day of testing. After 72 h of incubation the cell growth rate was evaluated by performing the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Working dilutions were freshly prepared on the day of testing. The maximal concentration of the solvent (dimethyl-sulphoxide, DMSO) never exceeded 0.5 %. After 72 hours of incubation the cell growth rate was evaluated by performing the MTT assay, which detects dehydrogenase activity in viable cells. The results are expressed as IC_{50} values.

Preparation of Ligands

N–salicylidene amino acids ligands were prepared according to the procedure.[29] Solid amino acids (2mmol, 147 mg Gly; 276 mg Met; 329 mg Phe) were initially dissolved in methanolic solution of KOH (2 mmol, 110 mg) and salicylaldehyde (2 mmol, 213 μL) was added dropwise. *N*-salicylidenecysteine was prepared *via* the reaction of equimolar amounts of L-cysteine (2 mmol, 242 mg) and salicylaldehyde (2 mmol, 213 μL) in ethanol (96 %, 70 mL) at 70 °C.[30,31] Ligand derived from salicylaldehyde and methionine was prepared by mechanochemical procedure.[32]

Preparation of Complexes

Complexes of the general formula $[Ru(bpy)_2L^{a–d}](OTf) \cdot nH_2O$ (**1a–1d**), where bpy = 2,2'–bipyridine and $L^{a,c,d}$ = potassium salts of *N*-salicylideneglycine (L^a), *N*-salicylidenemethionine (L^c), *N*-salicylidenephenylalanine (L^d) and L^b = *N*-salicylidenecysteine were prepared as described below. Starting complex compound *cis*- $[Ru(bpy)_2Cl_2 \cdot 2H_2O$ (0.19 mmol, 100 mg) was suspended in acetone (25 mL) and silver triflate (0.38 mmol, 98 mg) was added in portions. The reaction mixture was magnetically stirred protected from sunlight at room temperature during 1 h for complete removal of chlorides, after which the precipitate AgCl was filtered off. The acetone was completely removed from the filtrate under reduced pressure. Remained solid substance was suspended in methanol (30 mL) and further used for the synthesis of the complexes.[33] To the resulting solution Schiff base was added, $L^{a–d}$(0.19 mmol; 42 mg L^a; 45 mg L^b; 56 mg L^c; 59 mg L^d). The reaction mixture was refluxed for 3 h and then concentrated to a small volume (5 mL) after which diisopropyl ether (20 mL) was added dropwise. Red-to-brown solids were filtered off and dried under reduced pressure. Recrystallization was carried out from the ethyl methyl ketone and acetone for complex **1b**.

BIS(2,2'-BIPYRIDINE-κ^2N,N')[2-{(2-HYDROXYBENZYLIDENE)AMINO-κN}ACETATE-κ^2O]RUTHENIUM(II) TRIFLUOROMETHANESULFONATE–WATER (2/5) (1a)

120 mg (80 %); UV(MeOH) λ_{max} / nm: 202, 245, 292, 353 and 481 (log ε / dm^3 mol^{-1} cm^{-1}): 4.50, 4.35, 4.60, 3.82 and 3.77; IR (KBr) ν_{max} / cm^{-1}: 3446 (O–H), 1636 (C=N), 1606 (COO^-), 1396 (COO^-), 1285 (C–O), 767 (bpy), 640 (bpy); MS *m* / *z*: 592.0945 ($C_{29}H_{24}N_5O_3Ru^+$, 100 %). Anal. Calcd mass fractions of elements, *w* / %, for $C_{30}H_{29}F_3N_5O_{8.5}RuS$ (M_r = 785.71) are: C 45.86, H 3.72, N 8.91, Ru 12.86; found: C 45.78, H 3.04, N 9.17, Ru 12.41.

BIS(2,2'-BIPYRIDINE-κ^2N,N')[2-CARBOXY-2-{(2-HYDROXYBENZYLIDENE)AMINO- κN}ETHANETHIOLATE-κS]RUTHENIUM(II) TRIFLUOROMETHANESULFONATE (1b)

112 mg (75 %); UV(MeOH) λ_{max} / nm: 204, 244, 292, 340, 484 (log ε / dm^3 $mol^{-1}cm^{-1}$): 4.51, 4.28, 4.55, 3.80 and 3.75; IR (KBr) ν_{max} / cm^{-1}: 3430 (O–H), 1720 (C=O), 1606 (C=N), 1259 (C–O), 766 (bpy), 639 (bpy); MS *m* / *z*: 638.0829 ($C_{30}H_{26}N_5O_3SRu^+$, 100 %); Anal. Calcd mass fractions of elements, *w* / %, for $C_{31}H_{26}F_3N_5O_6S_2Ru$ (M_r = 786.77) are: C 47.26, H 3.45, N 8.89, Ru 12.85, found: C 47.46, H 3.35, N 9.41, Ru 13.35.

BIS(2,2'-BIPYRIDINE-κ^2N,N')[2-{(2-HYDROXYBENZYLIDENE)AMINO-κN}-4-(METHYLTHIO)BUTANOATE-κO]RUTHENIUM(II) TRIFLUOROMETHANESULFONATE–WATER (1/5) (1c)

103 mg (60 %); UV(MeOH) λ_{max} / nm: 203, 246, 290, 339 sh and 463 (log ε / dm^3 mol^{-1} cm^{-1}): 4.60, 4.48, 4.73 and 3.81; IR (KBr) ν_{max} / cm^{-1}: 3443 (O–H), 1630 (C=N), 1606 (COO^-), 1367 (COO^-), 1285 (C–O), 768 (bpy), 639 (bpy); MS *m* / *z*:

666.1108 ($C_{32}H_{30}N_5O_3SRu^+$, 100 %); Anal. Calcd mass fractions of elements, *w* / %, for $C_{33}H_{40}F_3N_5O_{11}RuS_2$ (M_r = 904.89) are: C 43.80, H 4.46, N 7.74, Ru 11.17, found: C 43.82, H 3.93, N 8.65, Ru 11.23.

BIS(2,2'-BIPYRIDINE-κ^2N,N')[2-{(2-HYDROXYBENZYLIDENE)AMINO-κN}-3-PHENYLPROPANOATE-κO]RUTHENIUM(II) TRIFLUOROMETHANESULFONATE–WATER (2/7) (1d)

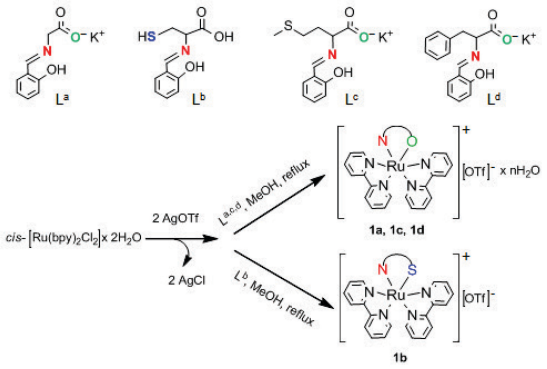
110 mg (65 %);UV (MeOH) λ_{max} / nm: 203, 243, 293, 360 and 492 (log ε / dm^3 mol^{-1} cm^{-1}): 4.58, 4.35, 4.59, 3.92 and 3.85; IR (KBr) ν_{max} / cm^{-1}: 3433 (O–H), 1631 (C=N), 1608 (COO^-), 1400 (COO^-), 1261 (C–O), 765 (bpy), 638 (bpy); MS *m* / *z*: 682.1420 ($C_{36}H_{30}N_5O_3Ru^+$, 100 %); Anal. Calcd mass fractions of elements, *w* / %, for $C_{37}H_{37}F_3N_5O_{9.5}RuS$ (M_r = 893.85): C 49.72, H 4.17, N 7.84, Ru 11.31, found: C 50.15, H 4.21, N 8.48, Ru 10.99.

RESULTS AND DISCUSSION

Synthesis

Ruthenium(II) complexes with amino acid Schiff bases were prepared by Ag^+ assisted substitution of chloro ligand[34] from *cis*-$[Ru(bpy)_2Cl_2] \cdot 2H_2O$ with Schiff bases in 1 : 1 molar ratio, in methanol, under reflux. All complexes were isolated as triflate salts upon addition of diisopropyl ether (Scheme 1.).

The formulation and characterization of the complexes, made by elemental analysis, mass spectrometry, infrared, electronic absorption spectroscopy and electrochemical data, refer to the octahedral coordination in cationic complex species by two bidentate diimine bipyridine ligands and one bidentate Schiff base. The collected data point to coordination mode of Schiff bases *via* nitrogen atom from azomethine group and oxygen atom from deprotonated carboxylate, for **1a**, **1c** and **1d.** In the case of **1b,** metal center is coordinated with S atom originating from deprotonated –SH group of amino acid moiety and azomethine nitrogen. The difference in the type of donor atoms lies primarily in the soft character of Ru(II) and its affinity for softer atoms, on the other hand in a different group that is deprotonated on the ligands. The three ligands $\mathbf{L^{a,c,d}}$ were prepared under basic conditions as salts thus forcing coordination *via* carboxylate oxygen,[35] unlike to the cysteine-bearing ligand, prepared as a neutral molecule. The acidic character of the –SH group resulted in S, N coordination of Ru(II) in a stable five-membered ring. Isolated complexes are dark red-brown substances, soluble in dimethylformamide, acetonitrile, dimethylsulfoxide, methanol and ethanol, moderately soluble in dichloromethane and water, and insoluble in non-polar organic solvents such as ether, hexane or toluene



Scheme 1. Synthetic pathway for the complexes **1a**–**1d**.

Elemental analysis of C, H, N and spectrophotometric determination of Ru content confirmed the composition and purity of the complexes. Mass spectra showed ruthenium isotopic distribution for molecular ion $[M(bpy)_2L]^+$ which correspond to **1a**–**1d.**

Spectroscopic Characterization

Infrared spectra of metal complexes exhibit broad bands in the range 3430–3470 cm^{-1} indicating the presence of OH group from Schiff bases and coordinated water molecules.[36,37] Coordination of Schiff bases *via* azometine nitrogen was confirmed through shift of asymmetrical stretching vibrations by 3–11 cm^{-1} to lower values compared to free ligands and appear at 1636–1604 cm^{-1} region. The important changes in the spectra of free Schiff bases ($\mathbf{L^a}$, $\mathbf{L^c}$ and $\mathbf{L^d}$) and corresponding complexes cover a range of carboxylate absorptions thus indicating coordination through deprotonated oxygen atom. In free ligands, absorptions at 1601–1618 cm^{-1} and 1375–1410 cm^{-1} region were assigned to the asymmetric and symmetric stretching vibrations of carboxylate, respectively. In spectra of respective complexes, the asymmetric frequencies increased while the symmetric frequencies decreased which strongly suggests the increase in metal-oxygen interaction since carboxylate group became more asymmetrical as the metal-oxygen interaction became stronger. It was found that complex compounds exhibit greater Δ(COO) separation for 10 to 20 cm^{-1} compared to the spectra of free ligands, thus confirming the monodentate binding mode *via* oxygen atom.[38] In the case of compound **1b**, disappearance of the band assigned to *ν*(S–H) stretching vibration in the spectra of free ligand *N*-salicylidenecysteine $\mathbf{L^b}$ at 2555 cm^{-1} indicates deprotonation of –SH group and coordination through the sulphur atom in the complex. Bands of the medium intensity in the 1460–1410 cm^{-1} region in the spectra of complexes originating from in-plane deformations of diimine rings are considered to be significantly non-affected by coordination. Bands around 1260 and 520 cm^{-1} arise from symmetrical stretching of S=O and C–S bonds of

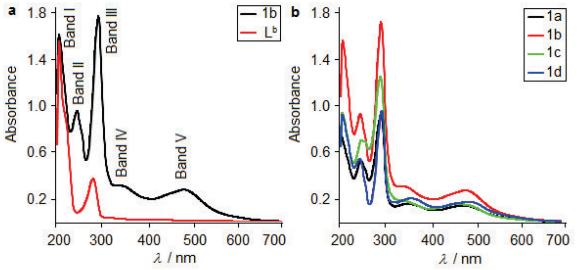


Figure 1. Electronic absorption spectra of a) **1b** and L^b; b) **1a**–**1d**; ($2.5 \cdot 10^{-5}$ M) in methanolic solution.

the free triflate anion, respectively. The electronic absorption spectra of complexes **1a**–**1d** in methanol solution exhibit five absorption bands in the 200–700 nm region. Bands I–III, located at the ultraviolet region were assigned to intraligand $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, the last attributed to transitions of the diimine ligand along with the free electronic pair of the azomethine nitrogen of the Schiff base. The absorptions in the visible region belong to allowed metal-to-ligand charge-transfer transitions. Two bands of charge-transfer transitions may be attributed to the presence of different acceptor orbitals.[34] Two well MLCT separated bands (IV and V) were observed in all complex compounds; the lower energy band correspond to the promotion of an electron from ligand easier to reduce (Figure 1).

The hydrolytic profile of **1a**, **1b**, **1c** and **1d** under physiological conditions showed inertness to hydrolysis within 24 hours, therefore, the biological activity against BSA and DNA, as well as antitumor activity is attributed to the species as formulated above.

Electrochemical Measurements

For Ru(II) polypyridine family, the most widely used electrochemical method has been cyclic voltammetry (CV) in non-aqueous aprotic solvents. Redox potentials for the **1a**–**1d** were recorded by cyclic voltammetry in acetonitrile so lution at a Pt working electrode with scan rate 100 mV s^{-1}

Table 1. Electrochemical data of the parent compound and **1a**–**1d**, utilizing cyclic voltammetry in acetonitrile solution with NBu_4PF_6 supporting electrolyte.

Compounds	(Ox_1 / Red_1) / V	(Ox_2 / Red_2) / V	(Ox_3 / Red_3) / V
1a	0.50 / 0.45*	0.72_{sh} / 0.67	0.86 / 0.78
1b	–	–	0.92 / 0.84
1c	0.54_{sh} / 0.50	0.72 / 0.61	0.88 / 0.79
1d	0.50 / 0.42	0.72 / 0.67	0.87 / 0.81
cis-$Ru(bpy)_2Cl_2 \cdot 2H_2O$	0.38 / 0.32	–	–

* poorly differentiated
sh shoulder

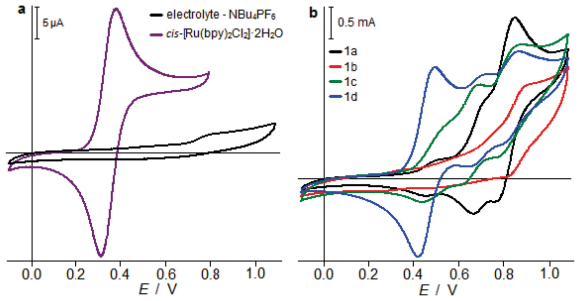


Figure 2. Cyclic voltammograms of a) *cis*-$[Ru(bpy)_2Cl_2] \cdot 2H_2O$ and supporting electrolyte NBu_4PF_6, b) **1a**–**1d**, in acetonitrile solution; working electrode Pt wire vs Ag/AgCl reference electrode; scan rate 100 mV s^{-1}; $E = 0.5 (E_{pa} + E_{pc})$; $\Delta E_p = E_{pa} - E_{pc}$.

and the data are summarized in Table 1. In this range, ligands are redox inactive, therefore the cathode and anode peaks can be attributed to electron transitions of complex species. The starting compound $Ru(bpy)_2Cl_2 \cdot 2H_2O$, under these conditions, showed a single-electron transition and electrochemical reversibility with a peak separation of 60 mV (Figure 2.) and an i_{pr} / i_{pf} value close to 1, indicating significant chemical reversibility.[39] The half wave redox potential $E_{1/2}$ was determined as $(E_{pa} + E_{pc}) / 2$, where E_{pa} and E_{pc} are the anodic and cathodic peak potentials, respectively. For irreversibile transitions $E_{1/2}$ could not be calculated.

Cyclovoltamograms of **1a**–**1d** (Figure 2.) show multiple redox pairs in the range 0–1 V that can be attributed to single–electron transitions $M \rightarrow L$ ($d\pi \rightarrow p\pi^*$) RuL^{3+} / RuL^{2+} where L represents both bpy and Schiff bases. These ligands increase electron density to Ru^{2+} therefore a turn back bonding of the metal to π^* unoccupied orbitales of bpy and salycilideneimines aromatic rings (π acceptors) occur.

The values of the first redox potentials for **1a**–**1d** relative to $Ru(bpy)_3^{2+}$ as the parent bipyridyl complex (+1.29 V) are shifted to less positive values due to the introduction of anionic ligand into the Ru(II) center. Although it is not always feasible to compare potentials reported in the literature on an absolute scale, the first anodic peak for **1a**, **1c** and **1d** appeared at about 0.5 V in correlation with the values found for heteroleptic complexes of general formula $Ru(bpy)_2L_2^+$ where L represents either *O*,*O* or *O*,*N* donor ligands.[8,40,41] On the other hand, $Ru(bpy)_2L_2^+$ where L presents *N*,*N* donor with an extended aromatic pattern show redox profile similar to parent $Ru(bpy)_3^{2+}$.[42]

Interaction with CT DNA

After success of cisplatin, numerous metal complexes, especially ruthenium-based compounds, with the ability to target DNA, have been designed. Although DNA is still considered the primary pharmacological target for Pt drugs in use, more and more potential targets for metal based drugs

are being revealed. Small molecules bind DNA in a reversible or irreversible mode, the latter implying covalent binding. Unlike cisplatin, which covalently binds nucleobases after hydrolysis of chloride, reversible bonding involves noncovalent interactions such as electrostatic bonding, intercalation, and major or minor groove binding. Numerous Ru(II) polypyridyl complexes containing extended planar aromatic system have shown the ability to intercalate DNA.[43–46] The most studied, $[Ru(bpy)_2dppz]^{2+}$ and $[Ru(phen)_2dppz]^{2+}$ where dppz = dipyridophenazine have high binding constants, $K_b = 10^6$–10^7 M^{-1}.[47,48] Interaction of **1a**–**1d** with CT DNA was followed by spectroscopic titration.

The binding constants K_b were calculated based on the equation (1):

$$\frac{[\mathrm{DNA}]}{(\varepsilon_a - \varepsilon_b)} = \frac{[\mathrm{DNA}]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{K_b(\varepsilon_b - \varepsilon_f)} \quad (1)$$

where ε_a, ε_f and ε_b stand for apparent extinction coefficients for particular measurements (A_{obs} / [DNA]), free complex and completely bound form, respectively.[49] By plotting [DNA] / ($\varepsilon_a - \varepsilon_f$) vs [DNA], K_b is obtained as the ratio of the slope and intercept (Figure 3). Spectrophotometric titration of complexes with increasing CT DNA concentration showed moderate hypochromism at MLCT bands and weak bathochromic which confirmed DNA intercalation.[50]

For the strongest polypyridyl Ru(II) intercalators (*vide infra*), Ru(dppz) unit is predominantly responsible for intercalation that occurs either through major or minor groove. In this sense, by comparing Ru(dppz) with Ru(L) unit of **1a**–**1d**, the weak intercalation in the DNA grooves correspond to K_b values of order 10^3 M^{-1} of **1a**–**1d** (Table 2). Since **1a**–**1d** have the same bipyridile intercalative ligand, the difference in K_b can be attributed to Schiff bases, with the same, unsubstituted salicylaldehyde, but different amino

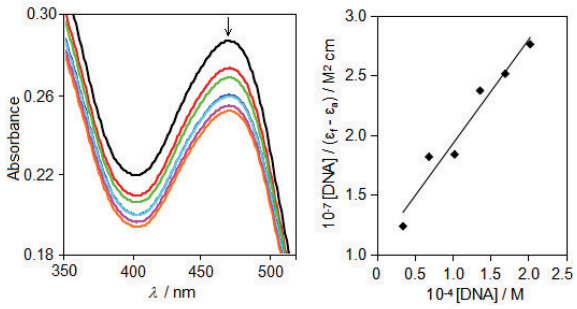


Figure 3. Spectroscopic titration of **1b** ($5 \cdot 10^{-5}$ M) with CT DNA (0–$2 \cdot 10^{-4}$ M), A_{260} / A_{280}=1.86) in 10 mM Tris-HCl buffer pH 7.42, T = 295 K, t = 2 min. *Inset*: Graphical determination of binding constant.

Table 2. Data on interaction of **1a**–**1d** with CT DNA.

	λ / nm	$\Delta\lambda$ / nm	K_b / M^{-1}	Linear equation / r^2 value
1a	468	2[b]	$7.28 \cdot 10^3$	$y = 1.87 \cdot 10^{-3} x + 2.57 \cdot 10^{-7}$; $r^2 = 0.99$
1b	469	1[b]	$8.32 \cdot 10^3$	$y = 8.74 \cdot 10^{-4} x + 1.05 \cdot 10^{-7}$; $r^2 = 0.95$
1c	466	3[b]	$1.67 \cdot 10^3$	$y = 9.55 \cdot 10^{-4} x + 5.74 \cdot 10^{-7}$; $r^2 = 0.97$
1d	478	0.2[b]	$1.55 \cdot 10^3$	$y = 9.65 \cdot 10^{-4} x + 6.24 \cdot 10^{-7}$; $r^2 = 0.99$

[b] batochromic

acids. Therefore, the contribution of the hydrogen bond to K_b via free carbonyl oxygen and OH group on Schiff bases can explain the differences in K_b which is for **1d** four to five times lower compared to **1a** and **1b**.

Interaction with BSA

In vitro protein inhibition assay of small molecules is almost inevitable part of the first approach to biological activity. The interaction of ruthenium(II) complexes **1a**–**1d** with BSA was investigated by spectrofluorimetry.

Intensity and the position of the emission maxima are directly related to the secondary structure of BSA.[51] Titration of aqueous solution of BSA with complexes resulted in quenching of intrinsic fluorescence (Figure 4). The quenching constant (K_{SV}) was determined graphically from Stern-Volmer equation (2) as the slope of [complex] vs I_0 / I plot.

$$\frac{I_0}{I} = 1 + K_{q\tau_0}[\mathrm{complex}] = 1 + K_{SV}[\mathrm{complex}] \quad (1)$$

where I_0 and I are fluorescence intensities in the absence and the presence of complex, respectively and K_q is the quenching rate constant, τ_0 is an average lifetime in the absence of the quencher ($\tau_0 = 10^{-8}$ s), [complex] is the concentration of complex and K_{sv} is the quenching constant. The quenching constant of complexes was 10^4 M^{-1} order, except for **1b** which was found to be 10^5 M^{-1} order demonstrating strong quenching of BSA fluorescence causing significant changes in polarity and conformation of tryptophan. The rate of quenching constant (k_q) was calculated as the K_{SV} / τ_0 ratio (τ_0 is an average lifetime of the fluorophore in the absence of the quencher which is usually near 10 ns). In case of **1b** k_q value was found to be 10^{13} M^{-1} and 10^{12} M^{-1} for **1a**, **1c** and **1d** which suggests static quenching mechanism. Expected maximum value of k_q for dynamic quenching is $2 \cdot 10^{10}$ M^{-1} s^{-1}.[52] The number of binding sites (n) and the binding constant (K_b) were determined from log[complex] *vs* log[($I_0 - I$) / I] plot (Figure 4). The number of binding sites was found to be near 1, indicating that the complexes prefer binding with BSA in 1 : 1 ratio.[53]

Table 3. Data on interaction of **1a**–**1d** with BSA.

	λ / nm	$\Delta\lambda$ / nm	K_{sv} / M^{-1}	K_b / 10^4 M^{-1}	*n*
1a	345	2.0[h]	9.21×10^4	0.58	0.77
1b	345	2.0[h]	1.52×10^5	3.80	0.89
1c	345	2.5[h]	8.04×10^4	0.17	0.68
1d	345	2.0[h]	8.12×10^4	0.10	0.63

[h] hypsochromic

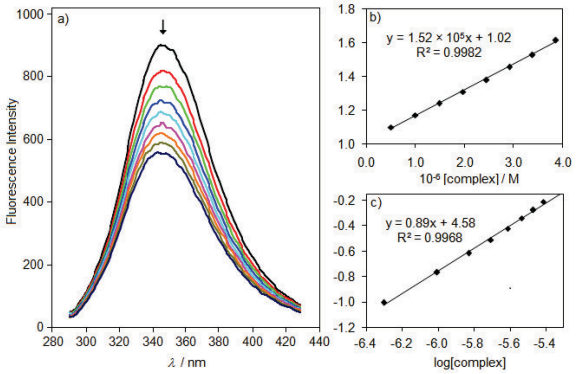


Figure 4. Interaction of **1b** with BSA: a) spectrofluorimetric titration of BSA with complex; b) graphical determination of Stern-Volmer constant; c) graphical determination of binding constant and number of binding sites.

Significant number of Ru(II) polypyridyl complexes showed strong emission intensity quenching upon interaction with serum albumins.[54–57] Among studied, special interest was devoted to $[Ru(bpy)_2(nap)][PF_6]$ and $[Ru(phen)_2(nap)][PF_6]$ where ligand nap = naproxen − NSAID drug. The results of interaction showed high binding affinity towards this biological target with quenching constants (K_{SV}) of 10^5 M^{-1} and binding constants (K) of 10^6 M^{-1} order, respectively. It is well known that serum albumins are major transporter for exogenous drugs and act important roles in their metabolism. Results on interaction of **1a**–**1d** compared to the literature data for the same Ru(II) polypyridyl unit can be associated with diverse molecular interaction, showing effective binding with the complexes.[58]

In vitro Antiproliferative Activity

The tested compounds **1a**–**1d** showed low to moderate antiproliferative activity only at the highest tested concentration. The most pronounced activity was demonstrated by compound **1a** and **1d** towards MCF–7 (breast adenocarcinoma) cells. It is noteworthy to mention that certain ruthenium(II) complexes incorporating amino acid component showed moderate to excellent *in vitro* antiproliferative activity towards S 180 (murine sarcoma

Table 4. IC_{50} values / μM of *in vitro* anticancer activity of **1a**–**1d**.

	IC_{50} / μm Cell lines		
	MCF-7	H460	SW 620
1a	32 ± 8	≥ 100	≥ 100
1b	≥ 100	≥ 100	≥ 100
1c	≥ 100	≥ 100	≥ 100
1d	26 ± 1	≥ 100	≥ 100

cancer), HEPG2 (human hepatoma) and K562 (human immortalized myelogenous leukemia) cell lines, ranging from 20 to 50 μM IC_{50} values.[59–61] Even though ruthenium(II) complexes derived from amino acid Schiff bases showed discrete *in vitro* antiproliferative activity, due to the amino acid component of the compounds, as the main building block of the proteins, their *in vivo* activity can be significantly different, as previously found with ruthenium complexes having antimetastatic activity.[6,62]

1a–**1d**, as coordinatively saturated and inert compounds, as well as many other Ru(II)-bipyridine compounds, demonstrated their antiproliferative activity by some mechanisms not–including strong DNA binding.[63] We compared IC_{50} values of **1a**–**1d** against MCF-7 with Ru(II) bipyridile complexes with different co-ligands. The values we obtained for **1a** and **1d** against MCF-7 cell line are lower compared to cisplatin (41.7 ± 1.5 μM) and quite comparable with activity of several Ru(II)-bipyridine compounds. Mononuclear and binuclear Ru(II) compounds $[Ru(bpy)_2(N–N)](CF_3SO_3)_2$, where 3,6-bis(2-pyridyl)-1,2,4,5-tetrazineis N–N chelator, showed similar IC_{50}= 25.4 ± 5.0 and 30.1 ± 12.5 μM and 30 % tumor reduction in the first *in vivo* study of Ru(II) polypyridyle compounds against breast cancer.[64,65] In the case of compounds with Schiff base ((phenylimino)methyl)phenol based ligand $[Ru(bpy)_2(N–O)](ClO_4)_2$ IC_{50} value against MCF–7 cell line is 17.99 ± 1.01.[66] The antiproliferative activity of several $[Ru(bpy)_2(N-N)]Cl_2$ compounds with extended π aromatic (N–N) ligands has been shown to depend on the surface area of N–N ligand; so for the $Ru(bpy)_3Cl_2$ is very high, IC_{50} 778. 3 ± 107.2 and down to 3.3 ± 1.2 for very extended pyridyle-pyridazine based ligand.[67]

CONCLUSION

In conclusion, novel heteroleptic Ru(II) complexes were added to the family of numerous Ru-bipyridile compounds. Although the composition of the complexes sufficiently differs through the imine ligand, derived from salicylaldehyde and dissimilar amino acids, no considerable difference in DNA and BSA binding was shown, even due to different coordination of RuN_5O (**1a**, **1c**, **1d**) and RuN_5S (**1b**). The

impact of coordination *via* cysteine sulfur (**1b**) showed the expected stabilization of the Ru(II) center and a significant shift of the oxidation peak towards more positive potential values. However, these complexes showed selective antiproliferative activity against MCF-7, H460 and SW 620 cell lines. While their activity is generally weak against tested cell lines with $IC_{50} \geq 100$ (**1b**, **1c**), **1a** and **1d** showed promising activity against breast cancer cells, similar to other heteroleptic Ru(II) bipyridyl complexes reported in the literature.

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