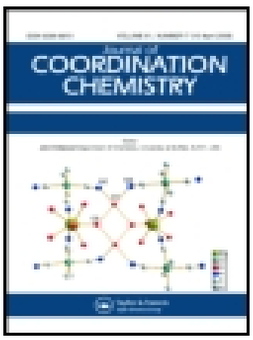

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Synthesis, characterization and antitumor activity of mononuclear and dinuclear ruthenium complexes

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Dinuclear ruthenium complexes $[Ru_2(bpy)_4BL](ClO_4)_2$ (**Ru-1**), where bpy = 2,2′-bipyridine and BL = 2,2′-((1E,1′E)-((E)-diazene-1,2-diyl-bis(2,1-phenylene))-bis(azanylyl-idene))bis(methanylylidene))diphenol (a bidentate bridging ligand), and mononuclear ruthenium complexes $[Ru(bpy)_2L](ClO_4)$ (**Ru-2**), where L = (E)-2-((phenylimino)methyl)phenol, were synthesized and characterized by elemental analysis and electrospray ionization mass spectrometry. Their photophysical and electrochemical properties were also studied. The cytotoxicity of the two complexes *in vitro* was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results indicated that **Ru-1** and **Ru-2** exhibited significant dose-dependent cytotoxicity to human breast cancer (MCF-7), gastric cancer (SGC-7901), cervical cancer (Hela) and lung cancer (A549) tumor cell lines. **Ru-1** showed excellent antitumor effects in a cellular study (IC_{50} values of 3.61 μM for MCF-7 human breast cancer cells *in vitro*). However, **Ru-2** exhibited the highest cytotoxicity to Hela cells; the IC_{50} value is 3.71 μM. The results reveal that **Ru-1** and **Ru-2** have obvious selectivity and might be a potential anticancer agent that could improve on the efficacy of common anticancer therapies.

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Keywords: Ru(II) Complexes; Spectral characteristic; Electrochemistry; Antitumor activity

1. Introduction

Development of more efficient anticancer drugs with better selectivity but less toxic side-effects is currently an area of hot research topic in bioinorganic chemistry [1, 2]. Since the discovery of cisplatin by Rosenberg in 1964, more and more attention has been paid to the metal complexes as potential anticancer drugs [3-5]. Cisplatin is still used today and is highly effective against testicular and ovarian carcinoma as well as bladder, head and neck tumors [6]. However, platinum-based anticancer chemotherapy is associated with severe side-effects because of poor specificity [7]. In the case of cisplatin, systemic toxicities like nephrotoxicity, neurotoxicity and ototoxicity inflict serious disorders or injuries on the patients during treatment, which badly restrict its efficacy [8]. Therefore, alternative metal compounds are presently being evaluated in clinical trials [9-12]. One of the most promising metals is ruthenium [13]. Ruthenium compounds are regarded as promising alternatives to platinum compounds and offer many approaches to innovative metallopharmaceuticals [14, 15]. The compounds are known to be stable and to have predictable structures both in the solid state and in solution: tuning of ligand affinities and accompanied by a steadily increasing knowledge of the biological effects of ruthenium compounds [16]. In year 1999, NAMI-A (ImH[*trans*-$RuCl_4$(DMSO)(Im)]) and in year 2003, KP1019 (InH[trans-$RuCl_4In_2$]) was successfully entered into phase I clinical trials for the treatment of metastatic tumors and colon cancers [17]. Now NAMI-A was successfully entered into phase II clinical trials for the treatment of non-small cell lung cancer [18]. Schiff bases have regularly been studied as ligands in coordination chemistry as a result of their good metal binding ability. The bidentate and multidentate Schiff bases with delocalized pi-orbitals are suitable ligands for the metals of biological importance, and the study of the chemical properties of such metal-Schiff base complexes also represents a good strategy for the design and synthesis of models of biological systems [19-22]. For example, the Ru(II) complex Ru(II)-2,6-bis(2,6-diisopropylphenyliminomethyl)pyridine exhibits very high cytotoxicity toward cancer cells [23],

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and Ru(II)-bis(arylimino)pyridine complexes with the co-ligands 1,10-phenanthroline (phen) and 2,2′-dipyridyl (bpy) can effectively inhibit the proliferation of the cancer cell lines with a low half-maximal inhibitory concentration (IC_{50}) [24]. On the side, Thota's group reported a series of novel mononuclear Ru(II) compounds and their *in vitro* antitumor activity toward the cell lines murine leukemia L1210, human lymphocyte CEM, human epithelial cervical carcinoma HeLa, BEL-7402 and Molt4/C8. Some of the complexes exhibited more potent antiproliferative activity against cell lines than the standard drug cisplatin [25]. These studies stimulate us to combine Ru metal with Schiff base to study its cytotoxicity toward cancer cells. So in this study, one dinuclear ruthenium complex $[Ru_2(bpy)_4BL](ClO_4)_2$ (**Ru-1**) [26, 27] and mononuclear ruthenium complex $[Ru(bpy)_2L](ClO_4)$ (**Ru-2**) (figure 1) were synthesized and characterized. The UV-vis absorption spectroscopy and electrochemical behavior of them were also studied. Moreover, their antitumor effects were investigated *in vitro* [28].

2. Experimental

2.1. *Materials and instrumentation*

All reagents and solvents were of commercial origin and used without further purification unless otherwise noted. Ultrapure Milli-Q water was used in all experiments. Dimethylsulfoxide (DMSO) and RPMI 1640 were purchased from Sigma. Hela (human cervical carcinoma), SGC-7901 (human gastric carcinoma), MCF-7 (human breast cancer) and A549 (human lung cancer) cell lines were purchased from the American Type Culture Collection. $RuCl_3 \cdot 3H_2O$, 2,2′-bipyridyl(bpy), (E)-2-((phenylimino)methyl)phenol (**L**), and the bridging ligand (BL), 2,2'-((1E,1'E)-(((E)-diazene-1,2-diylbis(2,1-phenylene))-bis-(azanylylidene))bis(methanylylidene))-diphenol were purchased from the Wuhan Shenshi Huagong, $[Ru(bpy)_2Cl_2] \cdot 2H_2O$ was synthesized and purified according to literature methods. Microanalysis (C, H and N) was conducted with a Perkin Elmer 240Q elemental analyzer. Electrospray ionization mass spectra were recorded with an LCQ system (Finnigan MAT, USA) using CH_3OH as the mobile phase. 300 MHz ^1H NMR spectra were recorded on a Bruker AM-300 NMR spectrometer using d_6-

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DMSO as solvent and TMS ($SiMe_4$) as an internal reference at 25 °C. Solution electronic absorption were recorded on a Shimadzu 3100 spectrophotometer in methanol. Cyclic voltammetry (CV) and differential pulse voltammetry (DVP) were done with a CHI 630E instrument in a three-electrode cell with a pure Ar gas inlet and outlet. The working and counter electrodes were Pt electrode, and the reference electrode was a saturated calomel electrode (SCE). The experiments were carried out in the presence of CH_3CN. DPV experiments were performed with a scan rate of 20 $mV \cdot s^{-1}$.

2.2. *Synthesis of Ru complexes*

2.2.1. [Ru(bpy)$_4$BL)](ClO$_4$)$_2$ (Ru-1). $AgNO_3$ (0.34 g, 2 mmol) was added to $[Ru(bpy)_2Cl_2]\cdot 2H_2O$ (0.52 g, 1 mmol) in absolute ethanol and the mixture was refluxed for 40 min. After cooling to room temperature, the solid residue (AgCl) was filtered off and discarded. To the filtrate, the ligand (BL) (0.11 g, 0.25 mmol) was added and refluxed for 6 h while magnetically stirred. After room temperature cooling, the solution was concentrated (10 mL) by evaporating most of the solvent. To the resulting solution, a saturated ethanolic solution of $NaClO_4$ (10 mL) was added, followed by water (100 mL). The solution mixture was left to settle at ambient temperature overnight. The brown precipitate was filtered off, washed with cold water and dried in vacuum. The purification of **Ru-1** was performed on a neutral aluminum oxide column. The first moving brown red band was eluted with acetonitrile containing 5% water. This was collected and evaporated, washed with cold water and dried in vacuum (yield 1.18 g, 82%). Data analysis: Elemental analysis calcd (%) for $C_{66}H_{50}Cl_2N_{12}O_{10}Ru_2$: C, 54.89; H, 3.49; N, 11.64. Found: C, 54.88; H, 3.50; N, 11.63. ESI-MS: m/z = 622.5 for $[\mathbf{Ru\text{-}1}]^{2+}$. UV-vis (in ethanol), λ_{max}(nm) (logε): 293 (2.48), 485 (0.44).

2.2.2. [Ru(bpy)$_2$L](ClO$_4$) (Ru-2). $AgNO_3$ (0.34 g, 2 mmol) was added to $[Ru(bpy)_2Cl_2]\cdot 2H_2O$ (0.52 g, 1 mmol) in absolute ethanol and the mixture was refluxed for 40 min. After cooling to room temperature, the solid residue (AgCl) was filtered off and discarded. To the filtrate, the

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ligand (L) (0.20 g, 1 mmol) was added and refluxed for 6 h while magnetically stirred. After room temperature cooling, the solution was concentrated (10 mL) by evaporating most of the solvent. To the resulting solution, a saturated ethanolic solution of $NaClO_4$ (10 mL) was added, followed by water (100 mL). The solution mixture was left to settle at ambient temperature overnight. The brown precipitate was filtered off, washed with cold water and dried in vacuum. The purification of **Ru-2** was performed on a neutral aluminum oxide column. The first moving brown red band was eluted with acetonitrile containing 5% water. This was collected and evaporated, washed with cold water and dried in vacuum (yield 1.4 g, 90%). Data analysis: Elemental analysis calcd (%) for $C_{33}H_{26}ClN_5O_5Ru$: C, 55.89; H, 3.70; N, 9.88. Found: C, 55.87; H, 3.71; N, 9.87. ESI-MS: m/z = 610.1 for $[\mathbf{Ru\text{-}2}]^+$.

2.3. *Methods*

2.3.1. Cytotoxicity assay *in vitro*. Standard MTT assay procedures were used. Cells were placed in 96-well microassay culture plates (8×10^3 cells per well) and grown overnight at 37 °C in a 5% CO_2 incubator. The complexes tested were then added to the wells to achieve final concentrations. Control wells were prepared by addition of culture medium (200 μL). The plates were incubated at 37 °C in a 5% CO_2 incubator for 48 h. On completion of the incubation, stock MTT dye solution (20 μL, 5 mg/mL) was added to each well. After 4 h, 150 mL DMSO was added to solubilize the MTT formazan. The optical density of each well was then measured with a microplate spectrophotometer at 490 nm. The IC_{50} values were determined by plotting the percentage viability versus the concentration and reading off the concentration at which 50% of the cells remained viable relative to the control. Each experiment was repeated at least three times to obtain the mean values. Four different tumor cell lines were the subjects of this study: MCF-7 (human breast carcinoma), SGC-7901 (human gastric carcinoma), Hela (huamn cervical cancer) and A549 (human lung carcinoma).

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2.3.2. Colony formation assay. The cells were plated in six well plates at 2000 cells per well. 24 h later, the complex was added to the plates. After treatment, fresh medium was applied to the plates. The cells were allowed to grow for ten additional days before staining with Crystal Violet (Sigma). All experiments were repeated at least three times, and similar results were obtained in each trial.

3. Results and discussion

3.1. *Synthesis and ^{1}H NMR*

Ru-1 and **Ru-2** were synthesized using the general procedure; the crystals of the complexes were not obtained. However, ^{1}H NMR spectra, ESI-MS and elemental analysis helped us to determine their structure. ^{1}H NMR spectra of the two complexes were recorded in d_6-DMSO. Since the electronic environments of many aromatic hydrogen atoms were very similar, their signals may appear in a narrow chemical shift range (7.0-9.0 ppm). In fact, the aromatic regions of the spectra were complicated, however, the number of the aromatic proton signals were consistent with the number of protons of molecular formula by integral area. The detailed ^{1}H NMR information of **Ru-1** and **Ru-2** are shown in figure 2. The ^{1}H NMR spectrum of **Ru-1** shows 11 sets of signals in the aromatic region, in fact there are 13 sets of signals theoretically, the partial overlapping of the signals made it less than theoretical peak. The signal assignment was rather straightforward by the comparison of chemical shifts with those of similar complexes [29-32]. The resonances at 8.64, 7.96, 7.61 and 7.40 ppm were assigned to the H protons of the 2,2′-bipyridine ring. The resonances at 8.70, 8.49, 8.01, 7.69, 7.44 and 7.19 ppm were assigned to the H protons of the phenyl ring. There was a singlet at 8.58 ppm, which indicated formation of -C=N in the Schiff base. The ^{1}H NMR spectrum of **Ru-2** shows 12 sets of signals in the aromatic region, in fact there are 14 sets of signals theoretically, the partial overlapping of the signals made it less than theoretical peak. The resonances at 8.81, 8.71, 7.96, 7.60, 7.50 and 7.36 ppm were assigned to the H protons of the 2,2′-bipyridine ring. The resonances at 8.74, 7.99, 7.95, 7.91, 7.82, 7.78 and 7.49 ppm were assigned to the H protons of the phenyl ring.

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3.2. *ESI-MS Spectroscopy*

The structures of **Ru-1** and **Ru-2** were further established by electrospray mass spectrometry (ESI-MS) in CH_3OH. This technique has proven to be very helpful for identifying metal complexes with high molecular masses. Dinuclear species **Ru-1** (figure 3), with one doubly (M^{++}) charged ion main peak obtained at m/z 623.2 $[Ru_2BL]^{2+}$, results from the total molecular weight 1444-$(ClO_4)_2$ to give a true mass of 1245 for which its doubly charged ion (M^{++}) is 622.5. Mononuclear complex **Ru-2** displayed one main peak at m/z 610.1 while there is no doubt that the peak at m/z 610.1 belongs to $[RuL]^+$ (*i.e.* [710-(ClO_4)-H^+] giving 609.5). It was suggested that both the dinuclear and mononuclear species should be stable in the solution.

3.3. *Electronic absorption spectra studies*

The UV-vis spectra of **Ru-1** and **Ru-2** are displayed in figure 4. **Ru-1** is characterized by two distinctive bands at 293 and 485 nm, and with a shoulder at about 340 nm, while the absorption spectrum of **Ru-2** is shown with complementary bands at 293 nm, 336 nm and 460 nm. The absorption bands below 350 nm are normally attributed to the overlapped π-π* intraligand transitions (ILCT) of the ligands while the broad absorption bands at 460 and 485 nm are ascribed to the metal-to-ligand charge transfer (MLCT) of the respective complexes [33, 34]. Compared with **Ru-2**, the MLCT band of **Ru-1** shows a significant broader band and slightly red-shifted with 25 nm (figure 4, inset) towards longer wavelength. This is due to the increase in the molecular area and the conjugation system of **Ru-1** as compared to **Ru-2**.

3.4. *Electrochemistry studies*

The characterization of **Ru-1** by cyclic voltammetry (CV) shows a reversible pattern with three redox peaks 0.51 V, 0.88 V and 1.13 V *vs.* SCE reference electrode in acetonitrile containing 0.1 M $[N(t\text{-}Bu)_4](ClO_4)$, as compared to only two peaks (0.71 V, 0.88 V) for **Ru-2**. The CV data are further complemented by the differential pulse voltammetry (DPV) peaks (figure 5(b)) for

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Ru-1 at 0.50 V, 0.79 V and 1.27 V, while for **Ru-2** at 0.70 V and 0.82 V, respectively. The three redox peaks observed for **Ru-1** are corresponding to the two one-step oxidation processes: $\mathbf{1}^{2+}\leftrightarrow\mathbf{1}^{3+}\leftrightarrow\mathbf{1}^{4+}$ and one for BL, which shows a very good electronic communication between the two ruthenium centers through the conjugated π-electron system as displayed by the weaker peak. The two peaks for **Ru-2** must be emanating from the ruthenium center and the ligand [35].

3.5. *In vitro* cytotoxicity

The MCF-7 (human breast cancer cell line), SGC-7901 (human gastric cancer cell line), Hela (human cervical cancer cell line) and A549 (human lung cancer cell line) were treated with different concentrations of ligands and complexes for 48 h. The cytotoxicity of these compounds towards the aforementioned cell lines was evaluated using the MTT method. Culture medium containing 0.05% DMSO was used as the negative control. After treatment of four cell lines for 48 h with **Ru-1** and **Ru-2** in a range of concentrations, the percentage inhibition of growth of the cancer cells was determined. The cell viabilities (%) *vs.* concentrations obtained with continuous exposure for 48 h are depicted in figure 6; the IC_{50} values are listed in table 1. The cytotoxicity of the complexes was found to be concentration-dependent. The cell viability decreased with increasing concentrations of both complexes. On comparison to ruthenium complexes, the ligands displayed the cytotoxicity at higher μM concentration. As shown in table 1, it was clear that **Ru-1** was active and exhibited low IC_{50} values (3.61±0.98 μM) to the MCF-7 cells at 48 h. However, **Ru-2** exhibited the highest cytotoxicity to Hela cells; the IC_{50} value is 3.71±3.5 μM. Moreover, the IC_{50} of **Ru-2** to SGC-7901 was lower than the $[Ru(bpy)_2(adpa)](PF_6)_2$ (bpy = 2,2′-bipyridine, adpa = 4-(4-aminophenyl)diazenyl-N-(pyridin-2-ylmethylene)aniline) which had been synthesized and reported by our group [36]. Dinuclear **Ru-1** displayed the cytotoxicity at lower μM concentration than $[(bpy)_2Ru(BL^{1-3})Ru(bpy)_2](PF_6)_4$ to MCF-7 [37]. On comparison with other ruthenium complexes (*e.g.* $[(bpy)_2Ru(L)Ru(bpy)_2]Cl_4$ (L = 1,6-bis(3-(*1H*-imidazo-[4,5-*f*][1,10]phenanthrolin-2-yl)-*9H*-carbazol-9-yl)hexane), IC_{50} >100 μM) [38], these reported ruthenium complexes displayed the cytotoxicity at lower μM concentration. These results reveal

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that **Ru-1** and **Ru-2** have obvious selectivity and might be a potential anticancer agent that could improve on the efficacy of common anticancer therapies [39].

3.6. *Colony formation assay*

The colony formation assay was performed to examine the effect of **Ru-1** on MCF-7 growth. As shown in figure 7, MCF-7 cells generated fewer colonies with increasing of **Ru-1** concentration, suggesting that colony was concentration dependent. Treated with 2.5 μM of complex, the colony number was about three times as much as 10 μM, treated with 30 μM of complex, the colony number was nearly 10% compared with control [40].

4. Conclusion

Two new Ru(II) complexes were synthesized and characterized using elemental analysis and electrospray ionization mass spectrometry. Absorption spectra and electrochemical behavior were also studied. **Ru-1** and **Ru-2** exhibited the spin-allowed singlet metal-to-ligand charge transfer transition at 485 and 460 nm. Moreover, **Ru-1** undergoes two metal-centered oxidation and one bridge ligand oxidation, while **Ru-2** undergoes one metal-centered oxidation and one ligand oxidation *vs.* a saturated calomel electrode. *In vitro* cytotoxic assays of the complexes were studied, and according to these studies, Ru(II) complexes showed significant dose-dependent cytotoxicity to breast cancer (MCF-7), gastric cancer (SGC-7901), cervical cancer (Hela) and lung cancer (A549) tumor cell lines, and **Ru-1** showed excellent antitumor effects in a cellular study to MCF-7. **Ru-2** exhibited the highest cytotoxicity to Hela cells; the IC_{50} value is 3.71 μM. The results reveal that **Ru-1** and **Ru-2** have obvious selectivity and might be a potential anticancer agent that could improve on the efficacy of common anticancer therapies. This work is ongoing in our group.

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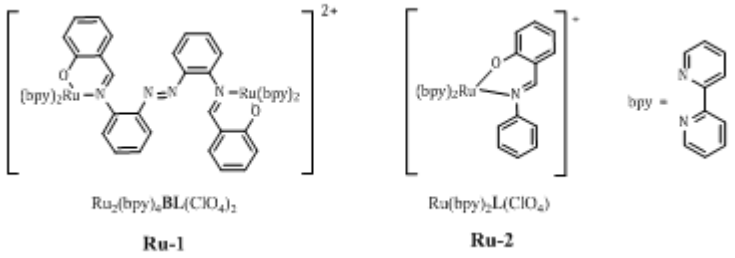

$Ru_2(bpy)_4BL(ClO_4)_2$ $Ru(bpy)_2L(ClO_4)$

Ru-1 **Ru-2**

Figure 1. Molecular structures of **Ru-1** and **Ru-2**.

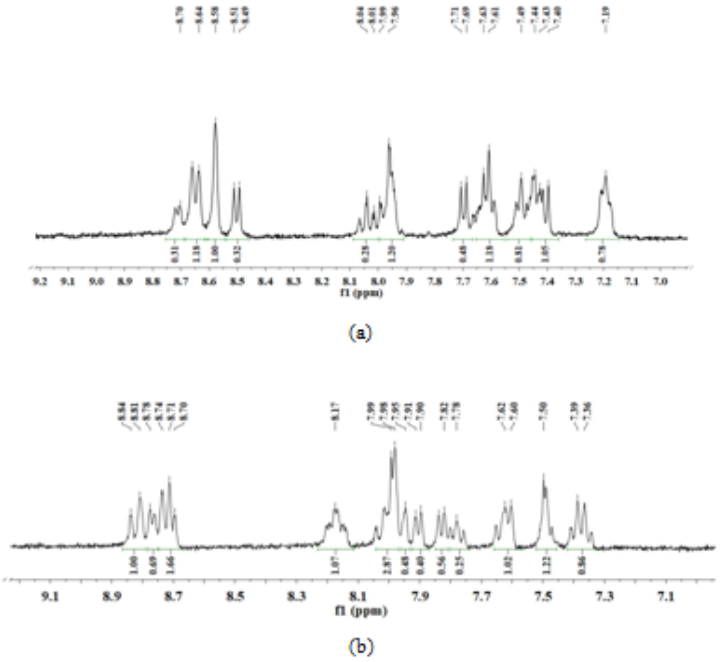

Figure 2. ^{1}H NMR spectrum of (a) **Ru-1** and (b) **Ru-2** in d_6-DMSO.

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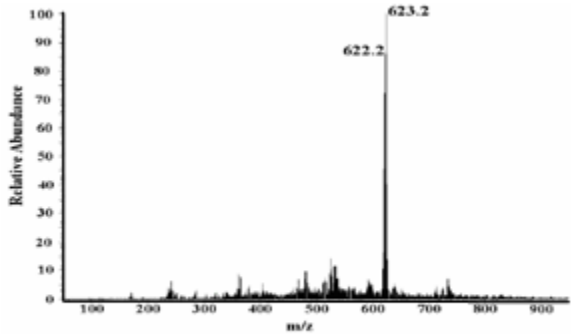

Figure 3. ESI-MS spectrum of **Ru-1** in methanol.

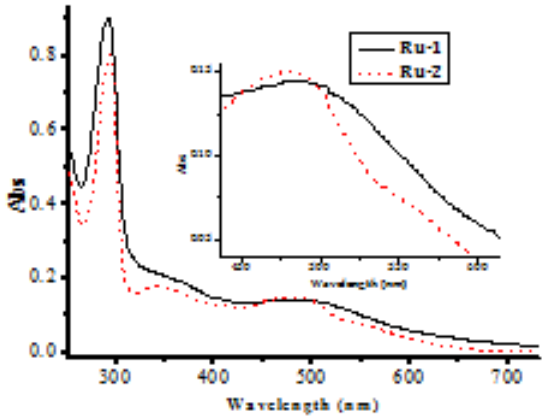

Figure 4. UV-Vis spectra complexes **Ru-1** and **Ru-2** (1×10^{-5} M) in methanol; the inset shows the broader band of **Ru-1**.

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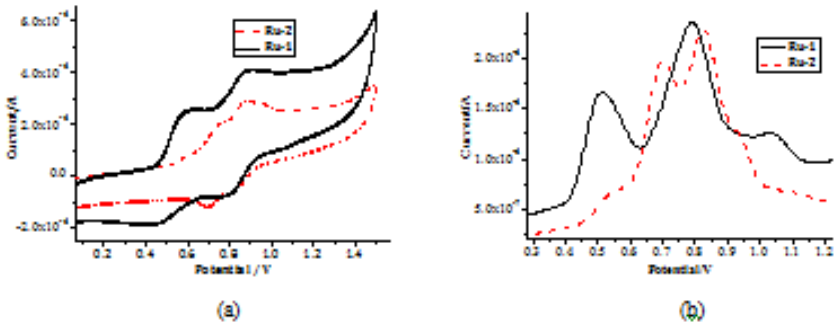

Figure 5. (a) CV and (b) DPV studies of **Ru-1** and **Ru-2** in acetonitrile.

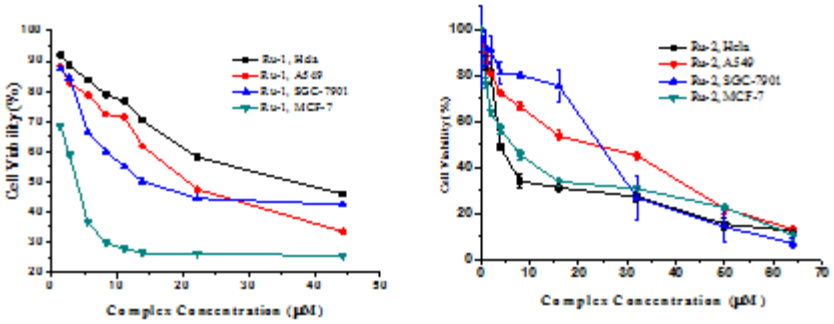

Figure 6. Cell viability of **Ru-1** and **Ru-2** toward proliferation of Hela, A549, SGC-7901 and MCF-7. Each point is the mean ± the standard error obtained from three independent experiments.

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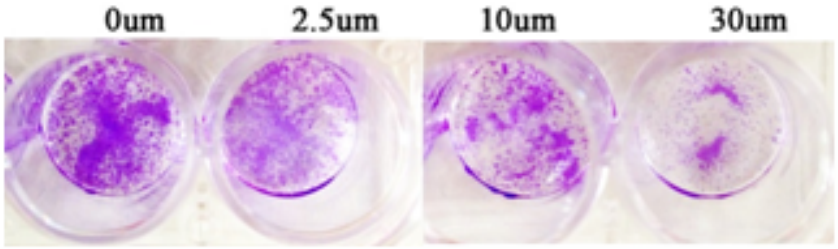

Figure 7. Antineoplastic activity of **Ru-1** to MCF-7 by colony formation assay.

Table 1. The IC_{50} (in μM) of ligands and Ru complexes.

Compound	Hela	A549	SGC-7901	MCF-7
BL	>100	>100	95.98±3.55	89.32±2.98
L	85.45±3.23	>100	>100	96.74±2.83
Ru-1	35.85±2.6	26.79±0.44	20.96±1.98	3.61±0.98
Ru-2	3.71±3.5	21.59±1.02	24.21±0.82	17.99±1.01

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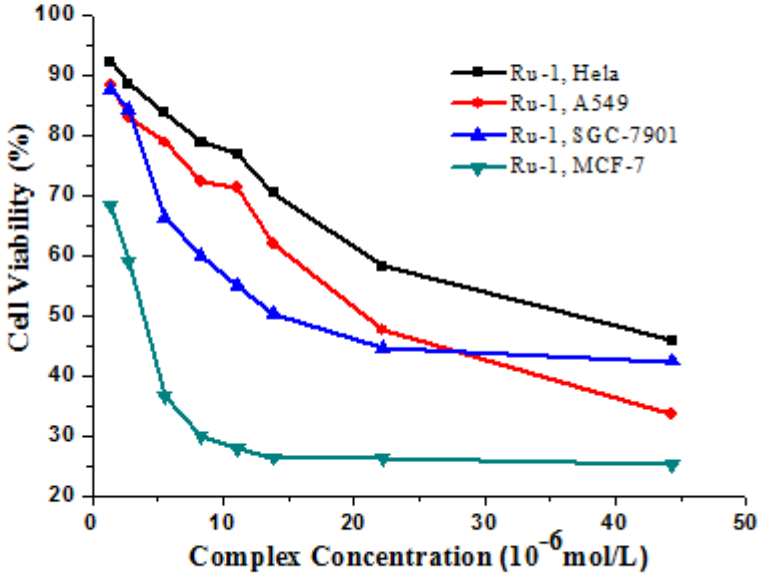
Graphical abstract



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