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DNA interaction, cytotoxicity, apoptotic activity, cell cycle arrest, reactive oxygen species and mitochondrial membrane potential assay induced by ruthenium(II) polypyridyl complexes

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ABSTRACT

Our previous studies showed that ruthenium(II) polypyridyl complexes can suppress the proliferation of BEL-7402 and MG-63 cells. In the present study, two active complexes **Ru-1** and **Ru-2** were identified to effectively inhibit BEL-7402, HeLa, MCF-7 and MG-63 cells growth. The cytotoxicity in vitro of the complexes towards the above cell lines were evaluated by MTT assay. The IC_{50} values range from 5.1 to 18.5 μM. Complex **Ru-2** shows higher cytotoxic activity than cisplatin towards selected cell lines under identical conditions. AO/EB and Hoechst 33258 staining and flow cytometry analyses indicate that **Ru-1** and **Ru-2** can induce apoptosis in BEL-7402 cells. In the assay of cell cycle arrest, **Ru-2** can induce G0/G1 phase arrest on BEL-7402 cells. The two complexes can enhance the level of ROS and decrease the mitochondrial membrane potential. **Ru-1** and **Ru-2** induced apoptosis in BEL-7402 cells through the mitochondrial pathway. In addition, the DNA-binding of these complexes with calf thymus was also investigated.

1. Introduction

Significant side effect, including nephrotoxicity, gastrointestinal toxicity, neurotoxicity, and totoxicity, low water solubility, and drug resistance have limited the clinical applications of cisplatin [1,2]. These limitations of cisplatin have motivated extensive investigations into alternative metal-based cancer therapies. Ruthenium complexes are widely believed as the substituents of cisplatin. Ruthenium complexes displayed diverse biological activities, including toxicity in mice, inhibition of the enzyme acetylcholinesterase, and bacteriostatic/bacteriocidal action against *Escherichia coli* and *Staphylococcus haemolyticus* [3]. Interest was stimulated by phase I clinical trials of two ruthenium complexes. Complex $(H_2im)[trans\text{-}Ru^{III}Cl_4(Him)(dmso)]$ (NAMI-A, Him = imidazole) was used as an antimetastatic drug, and $(H_2ind)[trans\text{-}Ru^{III}Cl_4(Hind)_2]$ (KP1019, Hind = indazole) was employed as an anticancer, and in particular colon carcinomas [4,5]. Recently, studies on the bioactivity of ruthenium complexes have been paid great attention and some interesting results have been obtained. $[Ru(phen)_2(p\text{-}MOPIP)]^{2+}$ can effectively inhibit the proliferation of HepG-2 cell line with a low IC_{50} value of 7.2 μM [6], Complex $[Ru(bpy)_2(dimb)]^{2+}$ (dimb = 6,6′-dimethyl-2,2′-bipyridine) is not cytotoxic in the dark, but when irradiated with visible light (>450 nm), the complex displays very high cytotoxicity with an IC_{50} value of 1.1 μM against A549 cancer cells [7], and $[Ru(dip)_2(dcdppz)]^{2+}$ (dcdppz = 7,8-dichlorodipyrido[3,2-*a*:2′,3′-*c*]phenazine) induces G0/G1 phase arrest on MG-63 cells [8]. $[Ru(R_2bpy)_2(dppz)]^{2+}$ can effectively inhibit the proliferation on HeLa cells [9]. ΔΔ-$Rubb_{16}$ entered the L1210 cells by passive diffusion (with a minor contribution from protein-mediated active transport), inducing cell death via apoptosis [10]. Based on our previous studies [11–14], we found that ruthenium complexes with polypyridyl ligands containing nitro-, amino- or hydroxyl group possess high cytotoxicity in vitro. In this report, two new ruthenium(II) polypyridyl complexes $[Ru(dmp)_2(NHPIP)](ClO_4)_2$ (**Ru-1**)

Abbreviations: NHPIP, 2-(3-nitro-4-hydroxylphenyl)imidazo[4,5-f][1,10]phenanthroline; AHPIP, 2-(3-amino-4-hydroxylphenyl)imidazo[4,5-f][1,10]phenanthroline; Dmp, 2,9-dimethyl-1,10-phenanthroline; DMSO, Dimethylsulfoxide; ES-MS, electrospray mass spectroscopy; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RPMI, Roswell Park Memorial Institute; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PI, propidium iodide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; DCF, 2,7-dichlorofluorescein; CTDNA, calf thymus DNA; MLCT, metal-to-ligand charge transfer transition.

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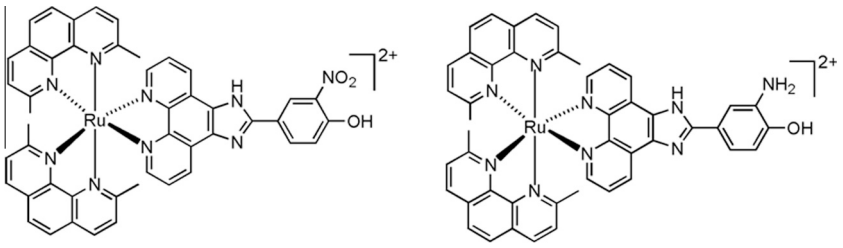

Ru-1 Ru-2

Scheme 1. The structures of **Ru-1** and **Ru-2**.

(dmp = 2,9-dimethyl-1,10-phenanthroline) and $[Ru(dmp)_2(AHPIP)](ClO_4)_2$ (**Ru-2**, Scheme 1) were prepared and characterized by elemental analysis, ES-MS and ^1H NMR. Their cytotoxicity in vitro was assessed by MTT assay (MTT = (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)). The apoptosis of BEL-7402 cells induced by the two complexes was also studied by fluorescence microscope and flow cytometry. The reactive oxygen species (ROS) and cell cycle arrest were analyzed by flow cytometry. The mitochondrial membrane potential induced by **Ru-1** and **Ru-2** was investigated under fluorescent microscope. The DNA-binding behaviors were studied by absorption spectra titration.

2. Experimental

2.1. Materials and methods

All reagents and solvents were purchased commercially and used without further purification unless otherwise noted. Ultrapure MilliQ water was used in all experiments. DMSO, 2,9-dimethyl-1,10-phenanthroline (dmp), and RPMI 1640 were purchased from Sigma. Cell lines of BEL-7402, HeLa, MCF-7 and MG-63 were purchased from the American Type Culture Collection. $RuCl_3{\cdot}3H_2O$ was purchased from the Kunming Institution of Precious Metals. 1,10-phenanthroline was obtained from the Guangzhou Chemical Reagent Factory.

Microanalyses (C, H, and N) were investigated with a Perkin-Elmer 240Q elemental analyzer. Electrospray ionization mass spectra (ES-MS) were recorded on a LCQ system (Finnigan MAT, USA) using methanol as mobile phase. The spray voltage, tube lens offset, capillary voltage and capillary temperature were set at 4.50 kV, 30.00 V, 23.00 V and 200 °C, respectively, and the quoted m/z values are for the major peaks in the isotope distribution. ^1H NMR spectra were recorded on a Varian-500 spectrometer with DMSO [D_6] as solvent and tetramethylsilane (TMS) as an internal standard at 500 MHz at room temperature.

2.2. Synthesis of complexes

2.2.1. Synthesis of $[Ru(dmp)_2(NHPIP)](ClO_4)_2$ (**Ru-1**)

A mixture of *cis*-$[Ru(dmp)_2Cl_2]{\cdot}2H_2O$ (0.312 g, 0.5 mmol) [15] and NHPIP (0.193 g, 0.5 mmol) [14] in ethylene glycol (20 cm^3) was refluxed under argon for 8 h to give a clear red solution. Upon cooling, a red precipitate was obtained by dropwise addition of saturated aqueous $NaClO_4$ solution. The crude product was purified by column chromatography on a neutral alumina with a mixture of CH_3CN–toluene (3:1, v/v) as eluent. The red band was collected. The solvent was removed under reduced pressure and a red powder was obtained. Yield: 71%. *Anal.* Calc. for $C_{47}H_{35}N_9Cl_2O_{11}Ru$: C, 52.57; H, 3.29; N, 11.74. Found: C, 52.38; H, 3.44; N, 11.56%. ES-MS [CH_3CN, m/z]: 873.4 ($[M–2ClO_4–H]^+$), 437.3 ($[M–2ClO_4]^{2+}$). ^1H NMR (500 MHz, DMSO-d_6): δ 8.97 (d, 4H, J = 8.5 Hz), 8.79 (s, 1H), 8.49 (t, 4H, J = 7.5 Hz), 8.30 (d, 2H, J = 8.5 Hz), 8.15 (d, 1H, J = 8.0 Hz), 8.04 (d, 2H, J = 8.0 Hz), 7.43 (d, 4H, J = 8.5 Hz), 7.21 (d, 2H, J = 4.0 Hz), 6.58 (d, 1H, J = 8.5 Hz), 3.46 (s, 1H), 2.00 (s, 6H), 1.77 (s, 6H).

2.2.2. Synthesis of $[Ru(dmp)_2(AHPIP)](ClO_4)_2$ (**Ru-2**)

This complex was synthesized with the identical method as described for **Ru-1**, with AHPIP [14] in place of NHPIP. Yield: 70%. *Anal.* Calc. for $C_{47}H_{37}N_9Cl_2O_9Ru$: C, 54.08; H, 3.57; N, 12.08. Found: C, 53.96; H, 3.71; N, 12.21%. ES-MS [CH_3CN, m/z]: 843.6 ($[M–2ClO_4–H]^+$), 422.3 ($[M–2ClO_4]^{2+}$). ^1H NMR (500 MHz, DMSO-d_6): δ 13.83 (s, 1H), 8.90 (d, 2H, J = 8.4 Hz), 8.80 (d, 2H, J = 8.0 Hz), 8.42 (dd, 4H, J = 8.0, J = 8.1 Hz), 8.23 (d, 2H, J = 8.8 Hz), 7.96 (d, 2H, J = 8.4 Hz), 7.46 (d, 2H, J = 8.8 Hz), 7.34 (d, 4H, J = 7.7 Hz), 7.30 (d, 1H, J = 4.5 Hz), 7.27 (d, 1H, J = 5.0 Hz), 6.81 (d, 1H, J = 8.0 Hz), 4.85 (s, 2H), 3.33 (s, 1H), 1.98 (s, 6H), 1.64 (s, 6H).

Caution: Perchlorate salts of metal compounds with organic ligands are potentially explosive, and only small amounts of the material should be prepared and handled with great care.

2.3. Viability assay in vitro

Standard 3-(4,5-dimethylthiazole)-2,5-diphenyltetraazolium bromide (MTT) assay procedures were used [16]. 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. Complexes tested were then added to the wells to achieve final concentrations ranging from 10^{-6} to 10^{-4} M. The culture medium and cisplatin are used as negative and positive controls. Control wells were prepared by addition of culture medium (100 μL). The plates were incubated at 37 °C in a 5% CO_2 incubator for 48 h. Upon completion of the incubation, stock MTT dye solution (20 μL, 5 mg mL^{-1}) was added to each well. After 4 h, buffer (100 μL) containing *N*,*N*-dimethylformamide (50%) and sodium dodecyl sulfate (20%) was added to solubilize the MTT formazan. The optical density of each well was then measured with a microplate spectrophotometer at a wavelength of 490 nm. The IC_{50} values were determined by plotting the percentage viability versus concentration on a logarithmic graph and reading off the concentration at which 50% of cells remain 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: BEL-7402 (Human hepatocellular carcinoma cell line), HeLa (Human vercix adenocarcinoma cell line), MG-63 (Human osteosarcoma cell line), and MCF-7 (Human breast cancer cell line).

2.3.1. Data analysis

All data was expressed as means ± SD. Statistical significance was evaluated by a t-test. Differences were considered to be significant when a P value was less than 0.05.