Journal of Inorganic Biochemistry 157 (2016) 62–72

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Synthesis, characterization, in vitro cytotoxicity and anticancer effects of ruthenium(II) complexes on BEL-7402 cells

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ARTICLE INFO

Article history:
Received 9 August 2015
Received in revised form 28 December 2015
Accepted 7 January 2016
Available online 22 January 2016

Keywords:
Ruthenium(II) polypyridyl complexes
Cytotoxicity in vitro
Reactive oxygen species
Mitochondrial membrane potential
Western blot analysis

ABSTRACT

Four new ruthenium(II) polypyridyl complexes $[Ru(dmb)_2(DQTT)](ClO_4)_2$ (**1**) (DQTT = 12-(1,4-dihydroquinoxalin-6-yl)-4,5,9,14-tetraazabenzo[b]triphenylene, dmb = 4,4′-dimethyl-2,2′-bipyridine), $[Ru(bpy)_2(DQTT)](ClO_4)_2$ (**2**) (bpy = 2,2′-bipyridine), $[Ru(phen)_2(DQTT)](ClO_4)_2$ (**3**) (phen = 1,10-phenanthroline) and $[Ru(dmp)_2(DQTT)](ClO_4)_2$ (**4**) (dmp = 2,9-dimethyl-1,10-phenanthroline) were synthesized and characterized by elemental analysis, ESI-MS, ^1H NMR and ^{13}C NMR. The cytotoxic activity in vitro of the complexes was evaluated against human BEL-7402, A549, HeLa, HepG-2 and MG-63 cancer cell lines by MTT (3-(4,5-dimethylthiazole)-2,5-diphenyltetrazolium bromide) method. The IC_{50} values of complexes **1**–**4** against BEL-7402 cells are 31.8 ± 1.0, 35.8 ± 1.6, 29.0 ± 0.8 and 25.0 ± 0.9 μM, respectively. The morphological apoptosis was investigated with AO/EB (acridine orange/ethidium bromide) and Hoechst 33258 staining methods. The DNA damage was assayed by comet assay. The inhibition of cell migration was evaluated by the wound healing assay. The levels of ROS (reactive oxygen species) and the changes of mitochondrial membrane potential were studied under fluorescent microscope. The percentages in the cells of apoptotic and necrotic cells and the cell cycle arrest were determined by flow cytometry. The expression of Bcl-2 family proteins was investigated by western blot analysis. The results show that the complexes induce BEL-7402 cells apoptosis through a ROS-mediated mitochondrial dysfunction pathway, which was accompanied by regulation of the expression of Bcl-2 family proteins.

1. Introduction

Carcinogenesis is thought to be prompted by changes to the DNA within cells and also by inhibition of growth suppressors, which, in turn, gives rise to the uncontrolled cell proliferation, invasion of surrounding and distant tissues, and ultimately leads to a risk of aggressive metastasis [1–3]. Cisplatin is one of the most widely used anticancer drugs. The clinical drawbacks of cisplatin are apparent, including the limited applicability, the acquired resistance, and the serious side effects, such as neurotoxicity and nephrotoxicity [4,5]. In the search for coordination compounds active against tumors and less toxic than cisplatin, ruthenium compounds emerge as the most promising with biological features including mechanism of action, toxicity and biodistribution which are very different from those of classical platinum compounds [6–12]. Three ruthenium(III) complexes entering clinical trials: NAMI-A [ImH][*trans*-$RuCl_4$(DMSO)(Im)], KP1019–[InH][*trans*-$RuCl_4(In)_2$] and NKP3019-Na[*trans*-$RuCl_4(In)_2$] (Im = imidazole, In = indazole) [13–20]. In recent years, the studies of ruthenium complexes on bioactivity have been made great progress and many ruthenium complexes display unique properties. The complex $[Ru(bpy)_2(adpa)](PF_6)_2$ (adpa = 4-(4-aminophenyl)diazenyl-N-(pyridin-2-ylmethylene)aniline) inhibits the cell growth at S phase [21]. Natarajan reported that $[Ru(H\text{-}Nap\text{-}etsc)Cl(CO)(PPh_3)_2]$ (H-Nap-etsc = 2-hydroxy-1-naphthaldehyde-4(*N*)-ethylthiosemicarbazone) induced apoptotic cell death via ROS hypergeneration and mitochondrial membrane damage. The studies on time dependent release of the complex from porous system embedded by mesoporous silica as the host material show that the main portion of the embedded complexes was released after 20 h and reached a maximum after 96 h [22]. $[Ru(phpy)(bpy)(dppn)]^+$ (bpy = 2,2′-bipyridine, dppn = benzo[*i*]dipyrido[3,2-*a*:2′,3′-*c*]phenazine) is 6 times more active than the platinum drug against HeLa cells [23]. Complex $[Ru(hdpa)_2(7\text{-}F\text{-}dppz)]^{2+}$ (7-F-dppz = 7-fluorodipyrido[3,2-*a*:2′,3′-*c*]phenazine) displays higher antitumor activity against HeLa cells with an IC_{50} value of 16.0 ± 2.48 μM [24]. The complex $[Ru(bpy)(phpy)(dppz)]^+$ (dppz = dipyrido[3,2-a:2′,3′-c]phenazine) was found to be rapidly taken up by cancer cells, and nearly 90% of the complex accumulated in the nuclei of cancer cells after a 2 h incubation [25]. Based on our previous work [26–28], we found that ruthenium(II) complexes show high inhibitory effect on the cancer cell growth. To further understand the mechanism

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http://dx.doi.org/10.1016/j.jinorgbio.2016.01.003
0162-0134/

of ruthenium complex inducing cancer apoptosis, in this report, a new ligand DQTT (DQTT = 12-(1,4-dihydroquinoxalin-6-yl)-4,5,9,14-tetraazabenzo[b]triphenylene) and its four ruthenium(II) complexes $[Ru(dmb)_2(DQTT)](ClO_4)_2$ (dmb = 4,4′-dimethyl-2,2′-bipyridine, **1**), $[Ru(bpy)_2(DQTT)](ClO_4)_2$ (bpy = 2,2′-bipyridine, **2**), $[Ru(phen)_2(DQTT)](ClO_4)_2$ (phen = 1,10-phenanthroline, **3**) and $[Ru(dmp)_2(DQTT)](ClO_4)_2$ (dmp = 2,9-dimethyl-1,10-phenanthroline, **4**, Scheme 1) were synthesized and characterized by elemental analysis, ESI-MS, ^1H NMR and ^{13}C NMR. The bioactivity of cytotoxic activity, cellular uptake, apoptosis, reactive oxygen species, mitochondrial membrane potential, cell cycle arrest and the expression levels of caspases and Bcl-2 family proteins were investigated in detail. The results show that the complexes induce BEL-7402 cell apoptosis through a ROS-mediated mitochondrial dysfunction pathway.

2. Experimental

2.1. Materials and methods

All reagents and solvents were purchased commercially and used without further purification unless otherwise indicated. Ultrapure MilliQ water was used in all experiments. DMSO, 2,2′-bipyridine, 4,4′-dimethyl-2,2′-bipyridine, 2,9-dimethyl-1,10-phenanthroline, and RPMI 1640 were purchased from Sigma. 1,10-phenanthroline was obtained from the Guangzhou Chemical Reagent Factory. Cell lines of HepG-2 (Human hepatocellular carcinoma), A549 (Human lung carcinoma), BEL-7402 (Hepatocellular), MG-63 (Human osteosarcoma) and HeLa (Human cervical cancer) were purchased from the American Type Culture Collection. $RuCl_3 \cdot 3H_2O$ was purchased from the Kunming Institution of Precious Metals.

Microanalysis (C, H, and N) was carried out with a Perkin-Elmer 240Q elemental analyzer. Electrospray ionization mass spectra (ESI-MS) were recorded on a LCQ system (Finnigan MAT, USA) using acetonitrile 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. The synthesis of ligand and complexes

2.2.1. 12-(1,4-dihydroquinoxalin-6-yl)-4,5,9,14-tetraazabenzo[b]triphenylene (DQTT)

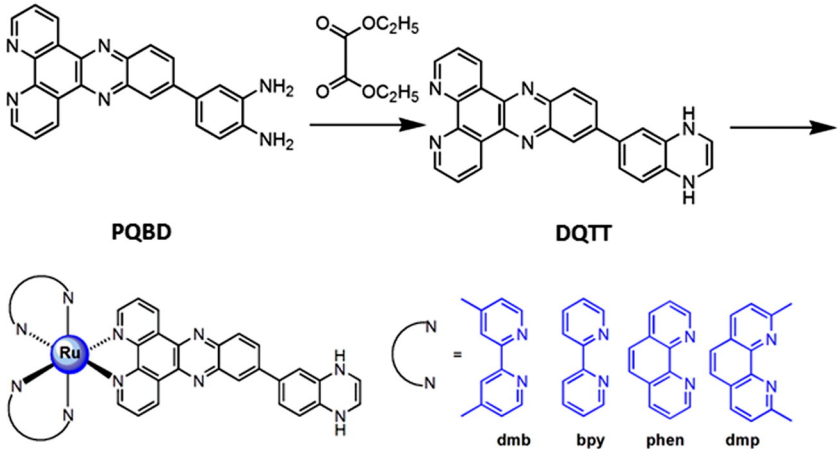
A mixture of 0.206 g (0.5 mmol) of PQBD (PQBD = 4-(4,5,9,14-tetraazabenzo[b]triphenylen-2-yl)benzene-1,2-diamine) [29], 105 μL (0.75 mmol) of diethyl oxalate was dissolved in 40 mL of glacial acetic acid and refluxed under nitrogen for 6 h. The yellow precipitate was washed with water (3 × 30 mL), and dried under vacuum. Yield: 80%. Anal. calc for $C_{26}H_{16}N_6$: C, 75.71; H, 3.91; N, 20.38. Found: C, 75.57; H, 3.99; N, 20.15%. ^1H NMR (DMSO-d_6): δ 9.57 (d, 1H, J = 6.0 Hz), 9.53 (d, 1H, J = 6.5 Hz), 9.21 (d, 2H, J = 6.0 Hz), 8.58 (d, 1H, J = 1.5 Hz), 8.45 (d, 2H, J = 6.0 Hz), 8.06 (d, 1H, J = 1.0 Hz), 7.97–7.93 (m, 2H), 7.77 (d, 1H, J = 2.0 Hz), 7.64 (d, 1H, J = 8.0 Hz), 7.12 (d, 1H, J = 3.5 Hz), 7.07 (d, 1H, J = 2.0 Hz), 3.38 (s, 2H). IR (KBr, cm^{-1}): 3060.2, 1682.1, 1615.5, 1574.9, 1501.4, 1476.3, 1449.7, 1360.6, 1288.3, 1213.8, 1127.4, 1073.0, 1047.5, 810.7, 759.7, 739.9, 700.9, 636.9. FAB-MS: m/z = 413 [M + 1].

2.2.2. Synthesis of $[Ru(dmb)_2(DQTT)](ClO_4)_2$ (1)

A mixture of cis-$[Ru(dmb)_2Cl_2] \cdot 2H_2O$ [30] (0.288 g, 0.50 mmol) and DQTT (0.206 g, 0.50 mmol) in ethylene glycol (20 mL) 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 neutral alumina with a mixture of CH_3CN-ethanol (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: 70%. Anal. calc for $C_{50}H_{40}N_{10}Cl_2O_8Ru$: C, 55.56; H, 3.73; N, 12.96%. Found: C, 55.43; H, 3.84; N, 12.85%. ^1H NMR (500 MHz, DMSO-d_6): δ 9.58 (dd, 2H, J = 8.5, J = 8.0 Hz), 8.73 (d, 5H, J = 8.5 Hz), 8.66 (d, 1H, J = 2.0 Hz), 8.58 (d, 1H, J = 7.0 Hz), 8.53 (d, 1H, J = 9.0 Hz), 8.26 (dd, 2H, J = 6.5, J = 5.5 Hz), 8.08 (s, 2H), 8.03–7.97 (m, 2H), 7.79 (d, 1H, J = 8.0 Hz), 7.64 (d, 4H, J = 6.0 Hz), 7.59 (d, 2H, J = 6.0 Hz), 7.42 (d, 2H, J = 5.5 Hz), 7.21 (d, 1H, J = 6.0 Hz), 3.40 (s, 2H), 2.56 (s, 6H), 2.48 (s, 6H). ^{13}C NMR (DMSO-d_6, ppm): 156.74, 156.54, 154.02, 153.70, 153.44, 151.51, 150.83, 150.57, 150.49, 150.39, 145.08, 142.89, 141.72, 140.75, 139.81, 133.47, 133.26, 132.78, 132.16, 130.51, 130.45, 130.33, 129.09, 128.94, 127.96, 125.47, 121.99, 21.25, 21.17. IR (KBr, cm^{-1}): 3389.7, 3067.2, 1617.7, 1548.5, 1524.4, 1485.8, 1444.1, 1364.9, 1317.1, 1285.5, 1240.9, 1197.2, 1033.2, 971.5, 929.1, 877.7, 823.4, 788.0, 739.6, 724.7, 623.3. ESI-MS (CH_3CN): m/z 880.4 ($[M–2ClO_4–H]^+$), 440.6 ($[M–2ClO_4]^{2+}$).

2.2.3. Synthesis of $[Ru(bpy)_2(DQTT)](ClO_4)_2$ (2)

This complex was synthesized in a manner identical to that described for **1**, with $[Ru(bpy)_2Cl_2] \cdot 2H_2O$ [30] in place of $[Ru(dmb)_2Cl_2] \cdot 2H_2O$. Yield: 71%. Anal. calc for $C_{46}H_{32}N_{10}Cl_2O_8Ru$: C, 53.91; H, 3.15; N, 13.67%. Found: C, 53.79; H, 3.01; N, 13.85%. ^1H NMR (500 MHz, DMSO-d_6): δ 9.62 (dd, 2H, J = 8.0, J = 8.5 Hz), 8.87 (dd, 4H, J = 8.5, J = 8.0 Hz), 8.67 (s, 1H), 8.60 (d, 1H, J = 9.0 Hz), 8.53 (d, 1H, J = 8.0 Hz), 8.24 (d, 4H, J = 8.5 Hz), 8.14 (t, 3H, J = 8.0 Hz), 8.04 (d, 3H, J = 6.0 Hz), 7.84 (d, 4H, J = 5.5 Hz), 7.76 (d, 1H, J = 6.0 Hz), 7.60 (t,



Scheme 1. The synthetic route of ligand and complexes.