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Research paper

A ruthenium(II) β-carboline complex induced p53-mediated apoptosis in cancer cells

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ABSTRACT

A ruthenium(II) β-carboline complex $[Ru(tpy)(Nh)_3]^{2+}$ (tpy = 2,2′:6′,2″-terpyridine, Nh = Norharman, **Ru1**) has been synthesized and characterized. This complex induced apoptosis against various cancer cell lines and had high selectivity between tumor cells and normal cells. In vivo examination indicated **Ru1** decreased mouse MCF-7 and HepG2 tumor growth. Signaling pathways analysis demonstrated that this complex induced apoptosis via the mitochondrial pathway, as evidenced by the loss of mitochondrial membrane potential (MMP, $\Delta\Psi_m$) and the release of cytochrome *c*. The resulting accumulation of p53 proteins from phosphorylation at Ser-15 and Ser-392 correlated with an increase in p21 and caspase activation. Taken together, these findings suggest that **Ru1** exhibits high and selective cytotoxicity induced p53-mediated apoptosis and may contribute to the future development of improved chemotherapeutics against human cancers.

1. Introduction

Cisplatin, a leading metal-based anticancer drug, mediates its anticancer effect by covalently binding to DNA to form adducts that interfere with transcription and DNA replication, thereby triggering programmed cell death [1]. However, significant side effects and drug resistance have limited its clinical applications and have also stimulated the development of non-platinum metal-based therapeutics [2–4]. Ruthenium complexes are attractive alternatives to platinum-based anticancer agents because of their rich synthetic chemistry, variable oxidation states that are accessible under physiological conditions, selective antimetastatic properties, multiple mechanisms of action that are distinct from those of platinum-based drugs and low systemic toxicity [5–7]. A number of ruthenium compounds have demonstrated promising anticancer activity, and some complexes exhibit a cytotoxic potency similar to or better than that of cisplatin Refs. [8–12].

Some previous works have showed that Ru(II) polypyridyl complexes with bioactive alkaloids as ligands offers new opportunities for the design of novel anticancer drugs with enhanced and targeted activity [13–17]. The β-carboline alkaloids are a class of synthetic and naturally occurring compounds that possess a large spectrum of important pharmacological properties including sedative, anxiolytic, antiviral, antimicrobial and antitumor activities. It has been reported that some β-carboline alkaloids can exert antitumor activities through multiple mechanisms, such as DNA-binding and inhibiting topoisomerases I and II, CDKs (cyclin-dependent s), and IκB kinases [18–20]. Due to our group's interest in β-carboline derivatives [21], a number of ruthenium complexes with β-carboline derivatives were synthesized [5,22]. These complexes exhibited cytotoxic potencies higher than that of cisplatin and inhibited cell growth through the induction of G0/G1-phase cell cycle arrest and apoptosis. However, the structure-activity relationships and the mechanism of the antitumor action by these complexes remain largely speculative.

It is noticed that most of reported Ru(II) complexes contain only bidentate ligands, and investigations of ruthenium complexes with tridentate ligands are rare [23,24]. In fact, molecular shape, among the various factors that contribute to stabilizing the metal complex on the DNA helix, appears to be the most significant [25]. $[Ru(tpy)_2]^{2+}$ (tpy = 2,2′:6′,2″-terpyridine) complexes are achiral and the two tpy units are perpendicular to each other, for which binding to DNA appears to be sterically possible. In addition, tridedante precursor $[Ru(tpy)]Cl_3$ exhibits more chelating sites when compared to bidedante precursor $[Ru(bpy)_2Cl_2]$ (bpy = 2,2′-bipyridine). Therefore, we explore a Ru(II) tridentate complex $[Ru(tpy)(Nh)_3]^{2+}$ (Nh = Norharman, **Ru1**, Fig. 1) as the new potentially antiproliferative agents. The antitumor activity and cellular uptake, as

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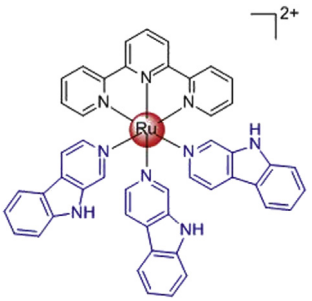

Fig. 1. Chemical structure of complex **Ru1**.

well as cell-cycle arrest and apoptosis signaling pathways were studied. The unique structure and characteristic of Ru(II) complex designed in this investigation may contribute to the future development of improved chemotherapeutics.

2. Experimental

2.1. Materials and measurements

All reagents were purchased from commercial sources and used without further purification unless otherwise specified. Chromatographic separations were performed on neutral aluminum oxide. All solvents were of analytical grade. All buffer components were of biological grade and used as received. $RuCl_3 \cdot nH_2O$ (Alfa Aesar), tpy (Alfa Aesar), cisplatin (Acros), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliu bromide (MTT Sigma), propidium iodide (PI, Sigma) and L-Tryptophan (Sigma) were used without further purification. Ru(tpy)Cl_3 [26] and β-carboline [27] were prepared according to previously reported methods. The Norharman and Ru(II) complexes were dissolved in DMSO immediately preceding the experiments; the calculated quantities of the drug solutions were then added to the appropriate medium to yield a final DMSO concentration of less than 1% (v/v).

Microanalysis (C, H, and N) was carried out using a Perkin–Elmer 240Q elemental analyzer. Electrospray mass spectra (ESI-MS) were recorded on an LCQ system (Finnigan MAT, USA). The expected and measured isotope distributions were compared. The ^{1}H NMR spectra were recorded on a Bruker AVANCE 400 spectrometer (400 MHz). All chemical shifts are reported relative to tetramethylsilane (TMS). The UV–Vis spectra were recorded on a Varian Cary 300 spectrophotometer.

2.2. Synthesis of [Ru(tpy)(Nh)3](CF_3SO_3)$_2$ (*Ru1*)

Ru1 (Fig. 1) was synthesized by reacting [Ru(tpy)]Cl_3 (0.440 g, 1 mmol) with $AgSO_3CF_3$ (0.825 g, 3.3 mmol) in ethanol (250 mL) for 12 h. After AgCl was removed by filtration, Norharman (0.6 g, 3.6 mmol) was added, and the mixture was refluxed for 3 days. The reaction mixture was evaporated under reduced pressure, and the crude product was purified by column chromatography on alumina with CH_3CN-toluene (2:1, v/v) as eluent. Yield: 52.7%. Anal. calcd for $C_{50}H_{35}F_6N_9O_6RuS_2$: C, 52.81%; H, 3.10%; N, 11.09%. Found: C, 52.75%; H, 3.18%; N, 11.02%. ESI-MS (CH_3OH): 419.2 ($[M\text{-}2SO_3CF_3]^{2+}$). ^{1}H NMR (400 MHz, d_6-DMSO) δ 11.58 (d, J = 11.2 Hz, 2H), 11.42 (s, 1H), 9.36 (d, J = 5.2 Hz, 2H), 8.97 (d, J = 5.3 Hz, 1H), 8.91–8.80 (m, 3H), 8.74–8.69 (m, 2H), 8.60 (s, 2H), 8.46 (s, 2H), 8.41 (d, J = 7.8 Hz, 1H), 8.30 (t, J = 7.8 Hz, 1H), 8.20–8.12 (m, 3H), 8.05 (t, J = 8.1 Hz, 1H), 7.96–7.90 (m, 3H), 7.89–7.85 (m, 1H), 7.80 (s, 1H), 7.77 (d, J = 6.2 Hz, 1H), 7.67 (d, J = 3.3 Hz, 1H), 7.59–7.46 (m, 4H), 7.40 (t, J = 4.9 Hz, 1H), 7.29–7.15 (m, 2H).

2.3. Cell culture conditions and MTT assay

HeLa, HepG2, Bel-7402, L-02, MCF-7, MCF-10A, HCT-116(p53−/−), HCT-116(p53+/+) and HEK-293 cells were maintained as monolayer cultures in DMEM. The A549 cells were maintained in RPMI 1640. Exponentially growing cells were seeded in triplicate into 96-well plates at 1×10^4 cells/well. After incubation for 24 h, the cells were treated with increasing concentrations of the tested complexes for 48 h. To stain the viable cells, 20 μL of MTT (5 mg/mL, Sigma) was added to each well. The cells were then incubated for 4 h at 37 °C. After the media had been carefully aspirated without disturbing the formed formazan crystals, the dye was dissolved in 200 μL DMSO. The absorbance of the samples was measured at 570 nm in an ELISA reader (BioTek Instruments, Winooski, VT). The IC_{50} value is defined as the compound concentration at which the absorption reading is reduced by 50% with respect to the controls. The IC_{50} values were determined by interpolation from dose–response curves.

2.4. Cell uptake

For uptake studies, exponentially growing cells were harvested, and the resulting single-cell suspension was plated in 100 mm tissue culture plates (Costar) at 1×10^5 cells/plate. After 24 h at 37 °C, the medium of subconfluent cells was replaced with fresh medium containing the **Ru1** at appropriate concentration. After 0.5, 1, 1.5 and 2 h of drug treatment, the cells' monolayers were washed three times with cold saline solution (0.9% NaCl). The cells were then harvested by cell scraper and were completely removed from the plates by additional washing with cold saline solution. The cell suspension was centrifuged at 800 *g* for 10 min at 4 °C, and the pellets were resuspended in an appropriate volume of Milli-Q water to obtain a homogeneous cell suspension. Aliquots were removed and sonicated for protein determination by the BCA (bicinchoninic acid) method using BCA protein assay reagent (Thermo). The remaining cell suspension was transferred into a glass tube containing 65% HNO_3 and was mineralized until complete drying at 120 °C. Dry ruthenium-containing materials were dissolved in 3 mL Milli-Q water of 2% HNO_3 [28]. The ruthenium content was measured using an Agilent inductively coupled plasma mass spectrometry (ICP-MS) 7700x. Data were reported as the means ± standard deviation (n = 3).

2.5. Cell cycle and Annexin V analysis

HeLa cells were treated with **Ru1** for various concentrations (1, 2 and 5 μM). The cells were harvested and centrifuged (5 min at 800 g) and fixed in 2 mL of 70% aqueous ethanol (v/v). After an incubation period of at least 12 h at −20 °C, cells were centrifuged (10 min at 800 g) and washed twice with ice-cold PBS. The cells were resuspended in 200 μL staining solution containing PI (10 μg/mL) and DNase-free RNase (100 μg/mL) and analyzed by a BD FACSCalibur™ cytometer (Becton Dickinson, Heidelberg, Germany). The number of cells analyzed for each sample was 10, 000, and the experiments were repeated at least three times under identical conditions. Data were collected by BD CellQuest™ Pro software and analyzed by ModFit LT 2.0 software.

Annexin V and propidium iodide (PI) co-staining of the apoptotic membranes was performed by using the annexin V–FITC apoptosis detection kit following the manufacturer's protocol (Sigma–Aldrich). For confocal microscopy, HeLa cells seeded into 35 mm glass-bottom dishes (Corning) were treated with **Ru1** (1 μM) for 24 h. After washed twice with PBS, the cells were stained with annexin V–FITC for 10 min. Cells were washed twice with PBS and then were stained with PI and observed immediately under a confocal laser scanning microscope (TCS SP5, Leica, Wetzlar,