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Effect of radiation on cytotoxicity, apoptosis and cell cycle arrest of human osteosarcoma MG-63 induced by a ruthenium(II) complex

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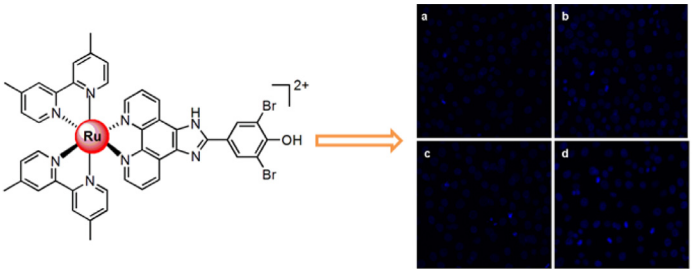
HIGHLIGHTS

- The cytotoxicity in vitro was investigated by CCK-8 method.
- The apoptosis assay was carried out with AO/EB staining and Hoechst 33258 methods.
- The cellular uptake was observed under confocal microscopy.
- Reactive oxygen species, mitochondrial membrane potential, cell cycle arrest were investigated.
- Western blot analysis was studied.

GRAPHICAL ABSTRACT

The cytotoxicity of Ru(II) complexes $[Ru(dmb)_2(DBHIP)](ClO_4)_2$ (**Ru1**) on MG-63 was evaluated by CCK-8 assay. The apoptosis induced by **Ru1** was investigated using fluorescent microscopy and flow cytometry. The cellular uptake, reactive oxygen species, mitochondrial membrane potential, cell cycle arrest and western blot analysis were investigated.



Apoptosis in MG-63 cells

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ABSTRACT

Radiation has large influence on the cytotoxicity, apoptosis and cell cycle arrest. The bioactivity of ruthenium(II) complex $[Ru(dmb)_2(DBHIP)](ClO_4)_2$ (**Ru1**) (DBHIP = 2-(3,5-dibromo-4-hydroxylphenyl)imidazo[4,5-f][1,10]phenanthroline) was investigated in the absence and presence of radiation. The cytotoxicity of **Ru1** against MG-63 cells was evaluated by CCK-8 method. **Ru1** shows high cytotoxicity upon radiation. Radiation can enhance the cytotoxicity of **Ru1** on MG-63 cells. The apoptosis was studied by Hoechst 33258 staining method and flow cytometry. The reactive oxygen species, mitochondrial membrane potential, cell cycle arrest and western blot analysis were investigated in detail. The complex induces the apoptosis in MG-63 cells through ROS-mediated mitochondrial dysfunction pathway.

Introduction

In recent years, great progress has been made in the studies on the bioactivity of ruthenium(II) complexes [1–13]. Complex $[Ru(dip)_2(1\text{-Py-}\beta C)]^{2+}$ {1-Py-βC = 1-(2-pyridyl)-β-carboline} displays higher

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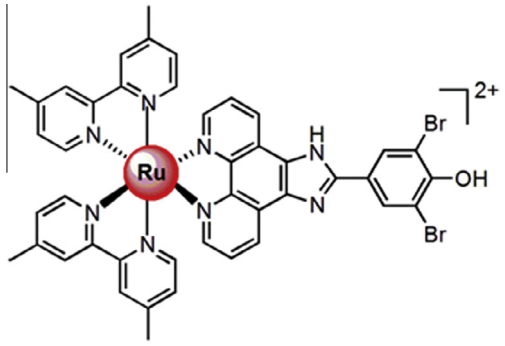

Scheme 1. The structure of **Ru1**.

cytotoxic activity than cisplatin against HepG-2, HeLa, and MCF-7 cells [14], $[Ru(phen)_2(paip)]^{2+}$ can effectively induce apoptosis in BEL-7402 cells [15], and $[(\eta^6\text{-}C_6Me_6)RuCl(dppn)]^{2+}$, bearing an arene ligand, that facilitates diffusion through cell membrane and thereby enhances cellular uptake, shows a high cell uptake of 1054.7 ng Ru/mg protein on HT-29 (colon cancer) cells [16]. $[Ru(phen)_2\text{-}p\text{-}MOPIP]^{2+}$ can induce mitochondria-mediated and caspase-dependent apoptosis in human cancer cells [17]. $[Ru(hdpa)_2(7\text{-}F\text{-}dppz)]^{2+}$ (hdpa = 2,2′-dipyridylamine, 7-F-dppz = 7-fluorodipyrido[3,2-*a*:2′,3′-*c*]phenazine) shows high antiproliferative activity toward HeLa [18]. $[Ru(dmb)_2(DBHIP)]^{2+}$ shows low cytotoxicity against BEL-7402, C-6, HepG-2 and MCF-7 cells [19]. In our previous work, we found that complex $[Ru(phen)_2(BHIP)]^{2+}$ (BHIP = 2-(3-bromo-4-hydroxyphenyl)[4,5]imidazo[1,10]phenanthroline) can effectively inhibit the cell growth of MG-63 cells with a low IC_{50} value of 10.3 μM [20]. Based on these studies, in order to obtain more insight into the studies of ruthenium(II) complexes on bioactivity, in this report, according to literature [19], we synthesized a ruthenium(II) complex $[Ru(dmb)_2(DBHIP)](ClO_4)_2$ (**Ru1**) (DBHIP = 2-(3,5-dibromo-4-hydroxylphenyl)imidazo[4,5-f][1,10]phenanthroline, Scheme 1). Osteosarcoma is the most common primary malignant bone cancer occurring in childhood [21]. Thus, the influence of the complex $[Ru(dmb)_2(DBHIP)](ClO_4)_2$ on osteosarcoma MG-63 cells was investigate. In the presence and absence of radiation, the cytotoxicity in vitro of **Ru1** on MG-63 cells was studied by CCK-8 method. The cellular uptake was observed under confocal microscope. The apoptosis by Hoechst 33258 staining method was investigated. The percentage of apoptotic and necrotic cells was determined by flow cytometry. The cell cycle arrest was analyzed by flow cytometry in PI (propidium iodide)-stained method. The reactive oxygen species, mitochondrial membrane potential and western blot analysis were studied.

Experimental

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. Osteosarcoma cells (MG-63) was purchased from the animal laboratory of Yat-Sen Sun University, Guangzhou. RPMI 1640 and 0.25% trypsin was purchased from Gibco Company, and fetal bovine serum (FBS) was obtained from Hyclone Inc., CCK-8 cell counting kit was purchased from Dojindo Co., Ltd., in Japan. Dimethyl sulfoxide (DMSO) was obtained from Sigma. Apoptosis-Hoechst 33258 staining kit was purchased from the biotechnology Research Institute R & D.

*Synthesis of complex **Ru1***

This complex was synthesized according to the literature [19].

Cell culture

MG-63 cells were grown in 25 mL flasks in RPMI 1640 media supplemented with penicillin and streptomycin (100 U/mL) and 10% of fetal bovine serum (FBS). Cells were incubated at the exponential growth phase at 37 °C in 5% CO_2.

*Effect of radiation with **Ru1** on the tumorigenicity of MG-63*

The cells (1×10^4 cells per well) were seeded into a six-well flask for 24 h, then the culture medium was replaced with fresh medium that contained 100, 50, 25 and 0 μM of **Ru1**, respectively. The flasks were radiated (Elekta Oncology Systems, UK) for 1 min. The culture media was removed, and fresh media was added. After 14 days of incubation, colonies were fixed with methanol and stained with Giemsa. The number of colonies containing at least 50 cells was determined, and the plating efficiency (PE) was calculated. PE = (number of clones in each group/number of cells inoculated in each group) × 100%.

*The median lethal concentration of **Ru1** toward MG-63*

The median lethal concentration of **Ru1** against MG-63 cells was assayed by CCK-8 method. In the determination of lethal concentration, a specified number (1×10^4 cells per well) of single cells were seeded into a 96 wells flask, after 24 h, the cells were treated with 100, 50, 25, 12.5, 6.25 and 0 μM of **Ru1** in the absence or presence of irradiation, respectively. After 24, 48, 72 and 96 h, 10 μL of CCK-8 solution (5 mg/mL) were added to each well at 37 °C for 4 h and sent to enzyme-linked immunosorbent assay and the values were recorded at 450 nm wavelength. The relative cell growth (%) related to controls was calculated using the following formula: [(test − blank)/(control − blank)] × 100%. The IC_{50} values were determined by plotting the percentage of viability versus concentration on a logarithmic graph and reading off the concentration at which 50% of cells remain viable relative to the control. Experiment was repeated three times to obtain the mean values.

Cellular uptake studies

MG-63 cells were placed in 24-well microassay culture plates (4×10^4 cells per well) and grown overnight at 37 °C in a 5% CO_2 incubator. Complex tested was then added to the wells. The plates were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Upon completion of the incubation, the wells were washed three times with phosphate buffered saline (PBS), after removing the culture mediums in the wells. The cells were visualized by confocal microscope.

Apoptosis assay with Hoechst 33258 staining method

MG-63 cells were seeded onto chamber slides in six-well plates at a density of 1×10^6 cells per well and incubated for 24 h. The cells were cultured in RPMI 1640 supplemented with 10% of FBS and incubated at 37 °C and 5% CO_2. The medium was removed and replaced with medium (final DMSO concentration, 0.05% v/v) containing the complex (25 μM) for 24 h in the absence or presence of irradiation. The medium was removed again. The cells were washed with ice-cold PBS, and fixed with formalin (4%, w/v). Cell nuclei were counterstained with Hoechst 33258 (10 mg/mL in PBS) for 10 min. The cell nuclei were observed and imaged by a fluorescence microscope.

Comet assay

DNA damage was investigated by means of comet assay. MG-63 cells in culture medium were incubated with 25 and 50 μM of **Ru1**