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Identification of Fluorescent Ruthenium Complexes Containing Imidazole Derivatives as a New Class of Mitochondria-Targeting Apoptosis Inducer by Living Cell Real-Time Imaging.

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Materials and methods

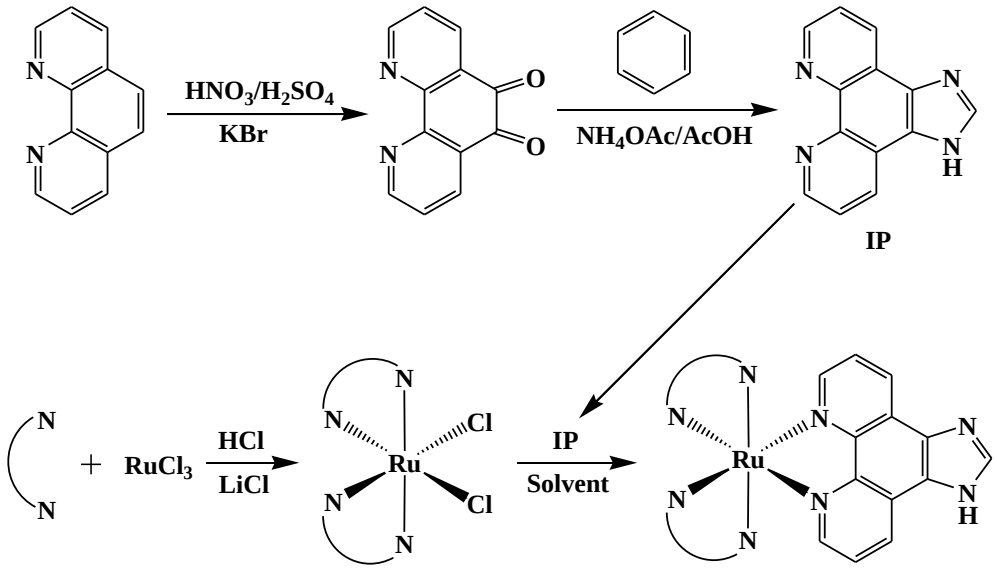
1. Instrumentation and Chemicals

All materials and solvents were purchased commercially and used without further purification unless otherwise noted. All of the antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA). Thiazolyl blue tetrazolium bromide (MTT), propidium iodide (PI), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), 4’,6-diamidino-2-phenyindole (DAPI) and MitoTracker Red CMXRos were obtained from Sigma. Dulbecco’s modified Eagle’s medium (DMEM), RPMI-1640 medium, bovine calf serum, and the antibiotic mixture (penicillin-streptomycin) were purchased from Invitrogen (Carlsbad, CA). Milli-Q water was used to prepare buffer solutions. The ligands biim [1] and IP [2] were prepared according to the literature procedures.

2. Experimental Procedure and Characterization Data for Products

2.1 Synthesis and characterization

A series of octahedral Ru complexes containing N,N-chelating ligands, $[Ru(L)_2IP]_2^+$ (IP = imidazol[4,5-*f*][1,10]phenanthroline; L = 2,2'-biimidazole (biim); 2,2-bipyridine (bpy); 1,10-phenanthroline (phen)) have been synthesized. The syntheses of 1, 2, and 3 are shown in **Scheme 1** and were achieved in a few steps.



Scheme 1. Schematic routes for synthesis of Ruthenium complexes

2.1.1 Synthesis of 2,2'-Biimidazole

The ligands 2,2' - Biimidazole and IP were prepared according to the literature procedures [1, 2].

2.1.2. *Cis*-$[Ru(biim)_2Cl_2] \cdot 2H_2O$

Cis-$[Ru(biim)_2Cl_2] \cdot 2H_2O$ were prepared and characterized according to the literatures [3, 4]

2.1.3. $[Ru(biim)_2IP](ClO_4)_2 \cdot 2H_2O$ (RuIP1)

$[Ru(biim)_2IP](ClO_4)_2 \cdot 2ClO_4$ were prepared and characterized according to the literatures [5]. 0.5 mmol of $[Ru(biim)_2Cl_2] \cdot 2H_2O$ (238 mg), 0.6 mmol of IP (133 mg) and 14 mL of ethylene glycol were added into a 50 mL three neck flask, the mixture was stirred and refluxed for 3 h under nitrogen protection, then cooled to room temperature and filtered. The solution was diluted with 30 mL of water, and saturated aqueous $NaClO_4$ solution was added dropwise. The product was collected by filtration,

purified by column chromatography on alumina using methanol as eluent, and dried in vacuo. Yield: 260 mg, 63 % on the base of $[Ru(biim)_2Cl_2]\cdot 2H_2O$, Calculated for $C_{25}H_{20}N_{12}Cl_2O_8Ru\cdot 2H_2O$ (%) : C 36.42, H 2.93, N 20.39. Found (%): C，36.49；H，2.98；N，20.41; ES- MS (*m/z*)：589.6 (M)$^+$ (calc：589.7)；294.8 (M)$^{2+}$ (calc：294.9).

Complexes $[Ru(bpy)_2IP](ClO_4)_2\cdot 2H_2O$ (RuIP2) and $[Ru(phen)_2IP](ClO_4)_2\cdot 2H_2O$ (RuIP3) were prepared by the same procedure as complex 1, using bpy and phen ligands instead of biim, with 75% and79% yield, respectively.

2.2. Cell lines and cell culture

Human cancer cell lines, including melanoma A375, hepatocellular carcinoma HepG2 and colorectal adenocarcinoma SW480, were purchased from American Type Culture Collection (ATCC, Manassas, VA). The normal fibroblast Hs68 and kidney HK-2 cells were also obtained from ATCC. All cell lines were maintained in either RPMI-1640 or DMEM media supplemented with fetal bovine serum (10%), penicillin (100 units/ml) and streptomycin (50 units/ml) at 37 °C in CO_2 incubator (95% relative humidity, 5% CO_2).

2.3. MTT assay

Cell viability was determined by measuring the ability of cells to transform MTT to a purple formazan dye [6]. Cells were seeded in 96-well tissue culture plates for 24 h. The cells were then incubated with the tested compounds at different concentrations for different periods of time. After incubation, 20 μl/well of MTT solution (5 mg/ml phosphate buffered saline) was added and incubated for 5 h. The medium was aspirated and replaced with 150 μl/well of acidic isopropanol (0.04 N HCl in isopropanol) to dissolve the formazan salt formed. The color intensity of the formazan solution, which reflects the cell growth condition, was measured at 570nm using a microplate spectrophotometer (SpectroAmax^{TM} 250).

2.4. Flow cytometric analysis

The cell cycle distribution was analyzed by flow cytometry as previously described [7]. Treated or untreated cells were trypsinized, washed with PBS and fixed with 75% ethanol overnight at -20°C. The fixed cells were washed with PBS and stained with propidium iodide (PI) (1.21mg/ml Tris, 700U/ml RNase, 50.1μg/ml PI, pH8.0) for 4 h in darkness. The stained cells were analyzed with Epics XL-MCL flow

cytometer (Beckman Coulter, Miami, FL). Cell cycle distribution was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA). Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak in the cell cycle pattern. For each experiment, 10,000 events per sample were recorded.

2.5. TUNEL assay and DAPI staining

Cells cultured in chamber slides were fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS. After then, the cells were incubated with 100 μl/well TUNEL reaction mixture containing nucleotide mixture and terminal deoxynucleotidyl transferase (TdT) for 1 h and 1 μg/ml of DAPI for 15 min at 37 °C respectively. The cells were then washed with PBS and examined under a fluorescence microscope (Nikon Eclipse 80i).

2.6. Evaluation of mitochondrial membrane potential

Cells in 6-well plates were trypsinized and resuspended in 0.5 mL of PBS buffer containing 10 μg/ml of JC-1. After incubation for 10 min at 37 °C in the incubator, cells were immediately centrifuged to remove the supernatant. Cell pellets were suspended in PBS and then analyzed by flow cytometry. The percentage of the green fluorescence from JC-1 monomers was used to represent the cells that lost $\Delta\Psi m$ [8].

2.7. Western blot analysis

Total cellular proteins were extracted by incubating cells in lysis buffer obtained from Cell Signaling Technology and protein concentrations were determined by BCA assay. SDS-PAGE was done in 10% tricine gels loading equal amount of proteins per lane. After electrophoresis, separated proteins were transferred to nitrocellulose membrane and blocked with 5% non-fat milk in TBST buffer for 1 h. After then, the membranes were incubated with primary antibodies at 1:1,000 dilutions in 5% non-fat milk overnight at 4°C, and then secondary antibodies conjugated with horseradish peroxidase at 1:2,000 dilution for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system (Kodak) [8]. To assess the presence of comparable amount of proteins in each lane, the membranes were stripped finally to detect the β-actin.

2.8. Living cell real time imaging

Cell mitochondria and nucleuses were stained with 50 nM MitoTracker Red CMXRos and 1 ug/ml DAPI, respectively for 20 min. After washing with PBS twice, cells were cultured in fresh medium on a thermo-cell culture FCS2 chamber of Carl Zeiss Cell Observer (Jena, Germany). Cell images were captured with a

monochromatic CoolSNAP FX camera (Roper Scientific, USA) and analysed by using AxioVision 4.2 software (Carl Zeiss).

2.9. Statistics analysis

All the data are expressed as mean ± SD. Differences between two groups were analyzed by two-tailed Student's t test. One-way analysis of variance (ANOVA) was used in multiple group comparisons. These analyses were carried out by SPSS 12.0. Difference with $P<0.05$ (*) or $P<0.01$ (**) was considered statistically significant.

Results

3.1 MTT assay.

As show in **Figure 1**, treatment of cells with RuIP1 resulted in time- and dose-dependent growth inhibition as examined by MTT assay. It appears good linear relationship between cell viability and concentration after a 72-h treatment with RuIP1.

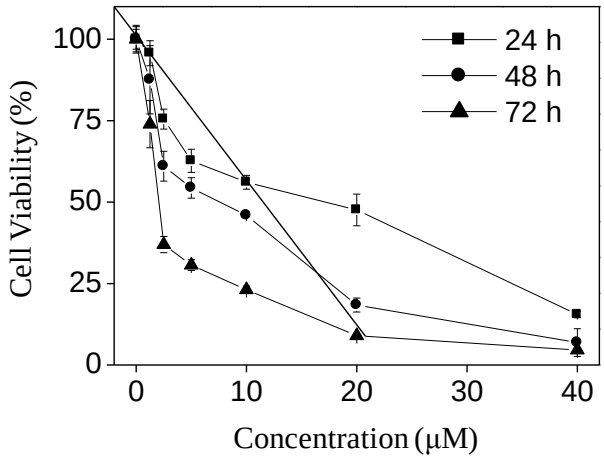


Fig. 1. Viability of A375 cells after treatment with different concentrations of RuIP1 for 24, 48, and 72 h, respectively.

3.2 Supplementary Video 1.

Live cell imaging of A375 cells upon incubation with 20 μM RuIP1. Cell morphology was captured by differential internal reflection fluorescence microscopy (Red: mitochondria; blue: nucleuses; green: RuIP1. Scale bar: 10 μm. Time presented as hr:min:sec.

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