Electronic Supporting Information

for

Ruthenium Complexes Containing Bis-benzimidazole Derivatives as a New Class of Apoptosis Inducers

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1. Materials and methods

1.1. Synthesis and characterization

1.1.1. Synthesis of bbp and 6-Mebbp

The ligands bbp and 6-Mebbp were prepared according to the literature procedures[1].

1.1.2. Ru(III)(bbp)Cl_3 (1) [2, 3]

A solution of $RuCl_3$•$3H_2O$ (0.2615 g, 1.0 mmol) in ethanol (20 ml) was added to a hot solution of bbp (0.311 g, 1.0 mmol) in ethanol (30 ml). The resulting brown solution was heated under reflux for 3 h and then cooled to room temperature. The brown solid was filtered, washed with ethanol and ether, and dried in a vacuum for 1 day. Yield 0.413 g, 79.1%. (Found: C, 41.0; H, 3.3; N, 12.5. Calc. for $C_{19}H_{13}Cl_3N_5Ru$•$2H_2O$: C, 41.1; H, 3.2; N, 12.6%). ESI-MS: *m*/*z* 483.4 (M -Cl). UV-Vis (λ (nm), ε/10^4 (M^{-1} cm^{-1}): 328 (1.50), 578 (0.20).

1.1.3. Ru(III) (bmbp)Cl_3 (2)

It was prepared according to the same procedure as in **1** with 6-Mebbp (0.339 g, 1.0 mmol) in place of bbp. Yield 0.382 g, 70.3%. (Found: C, 44.6; H, 3.4; N, 12.5. Calc. for $C_{21}H_{17}Cl_3N_5Ru$•H_2O: C, 44.7; H, 3.4; N, 12.4%). ESI-MS: *m*/*z* 511.5 (M -Cl). UV-Vis (λ (nm), ε/10^4 (M^{-1} cm^{-1}): 335(3.51), 578 (0.35).

1.1.4. [Ru(II) (bbp)(phen)]$^{2+}$ (1a)[4]

Phen (0.045 g, 0.25 mmol) in DMF (5 ml) was added to compound **1** (0.1296 g, 0.25 mmol) dissolved in hot DMF (120 ml). The mixture was heated in continuous reflux for 5 h under a nitrogen atmosphere and the colour of the solution changed from brown to red. Then the solution was cooled to room temperature. A red

precipitate was obtained by dropwise addition of a saturated aqueous $NaClO_4$. The product was purified by column chromatography on alumina using toluene-methanol as eluent and then dried in vacuo. Yield 0.085 g, 40.3%. (Found: C, 51.0; H, 2.9; N, 13.6. Calc. for $C_{31}H_{21}Cl_2N_7O_4Ru$: C, 51.1; H, 2.9; N, 13.5%). ESI-MS: *m*/*z* 628.4 (M $-ClO_4$). ^{1}H NMR (DMSO-d_6, δ ppm): 11.3 (d, 2H), 9.02 (d, 1H), 8.48-8.42 (m, 2H), 8.36 (d, 1H), 8.31-8.23 (m, 4H), 8.13 (d, 1H), 7.65 (q, 1H), 7.51 (d, 1H), 7.43 (d, 2H), 6.81 (t, 2H), 6.58 (t, 2H) and 5.86 (d, 2H). UV-Vis (λ (nm), $\varepsilon/10^4$ (M^{-1} cm^{-1}): 266 (4.93), 351 (3.60), 489 (1.15).

1.1.5. [Ru(II)(6-Mebbp)(phen)]$^{2+}$ (2a, RuBmP)

2a was prepared according to the same procedure as in **1a** with **2** (0.14 g, 0.25 mmol) in place of **1**. Yield 0.075 g, 35.2%. (Found: C, 52.4; H, 3.0; N, 12.9. Calc. for $C_{33}H_{23}Cl_2N_7O_4Ru$: C, 52.5; H, 3.0; N, 13.0%). ESI-MS: *m*/*z* 656.4 (M -ClO_4). ^{1}H NMR (DMSO-d_6, δ ppm): 9.05-8.99 (m, 1H), 8.36-8.46 (m, 4H), 8.28-8.12 (m, 4H), 7.65 (t, 1H), 7.51 (d, 1H), 7.29 (d, 1H), 7.20 (s, 1H), 6.63 (d, 1H), 6.40(d, 1H), 5.74 (q, 1H), 5.61 (d,1H), 2.18 (s, 3H) and 1.96 (s, 3H). UV-Vis (λ (nm), $\varepsilon/10^4$ (M^{-1} cm^{-1}): 267 (3.70), 344 (3.20), 525 (1.02).

1.1.6. [trans-Ru(III)Cl_4(Me_2SO)$_2$][(Me_2SO)$_2$H][5]

1.0 g of $RuCl_3$•$3H_2O$(0.0038 moles) was suspended in 30 ml ethanol and underwent reflux heating for three hours to obtain a dark green solution. the solution was filtered on paper to remove possible traces of undissolved solid; it was then concentrated by using a rotary evaporator up to 1/10 of the initial volume; therefore, 1 ml of aqueous concentrated HCl, 37% and 2 ml of DMSO were added and the mixture obtained in this way was kept at a temperature of 80 °C for about 15 minutes, up to obtaining a bright orange solution.

After cooling of the mixture to room temperature and after addition of 10 ml acetone, the product, after setting up, separated from the solution in the form of red-orange crystals; the formation of these crystals was accelerated by the addition of a few drops of ethyl ether. The crystals were then collected on a filter, washed with cold acetone (20 ml), then with ethyl ether (10 ml) and finally dried under vacuum at room temperature. 1.3 g of the final product with a 62.5% yield was obtained.

1.1.7. [H_2im][trans-Ru(III)Cl_4(dmso-S)(Him)](NAMI-A)[5]

1.0 g (0.0018 moles) of the [trans-Ru**(III)**$Cl_4(Me_2SO)_2$][$(Me_2SO)_2H$] complex prepared as described in 1.1.6., was suspended in acetone (20 ml) at room temperature. After the addition of 0.49 g (0.0072 moles) of imidazole, the mixture was kept under stirring for 4 hours; during this time the colour of the precipitate gradually changed from orange to brick red. After being collected on a filter and washed with acetone (10 ml) and therefore with ethyl ether (10 ml), the product was dried under vacuum at room temperature or in a oven at 60°C for a few hours. Hence, 0.70 g of final product were obtained with a 85.9% yield. ESI-MS: m/z = 389.7 [Ru**(III)**Cl_4(DMSO)(Him)]$^-$, m/z = 244 [Ru**(III)**Cl_4]$^-$.

1.1.8. Stability of Ru complexes in DMSO

The stability of the complexes in DMSO was examined by UV-Vis spectrometry. After the complexes were dissolved in DMSO, UV–Vis absorption spectra were recorded at different time points respectively.

1.2. Cell lines and cell culture

Human cancer cell lines, including A375 melanoma, Hela cervical carcinoma, MCF-7 breast adenocarcinoma, PC-3 prostate cancer, Neuro-2a neuroblastoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The normal Hs68 fibroblast 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).

1.3. 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).

1.4. 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) (1.21 mg/ml Tris, 700 U/ml RNase, 50.1 μg/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.

1.5. 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).

1.6. 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 $\Delta\Psi m$[6].

1.7. Measurement of superoxide generation (DHE assay).

After treatment, cells were incubated with DHE at a final concentration of 10 μM for 25 min at 37℃. Then, cells were trypsinized, centrifuged to remove the supernatant and resuspended in PBS. The level of superoxide was measured by flow cytometry.

1.8. Determination of activities of caspase-3, caspase-8 and caspase-9

Caspase activity was measured as previously described[8]. Harvested cell pellets were suspended in cell lysis buffer and incubated on ice for 1 h. After centrifugation at 11,000 × g for 30 min, supernatants were collected and immediately measured for protein concentration and caspase activity. Briefly, cell lysates were placed in 96-well plates and then specific caspase substrates (Ac-DEVD-AMC for caspase-3, Ac-IETD-AMC for caspase-8 and Ac-LEHD-AMC for caspase-9) were added. Plates were incubated at 37℃ for 1 h and caspase activity was determined by fluorescence intensity with the excitation and emission wavelengths set at 380 and 440 nm, respectively.

1.9. Real-time living cell 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).

1.10. Statistics analysis

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

2. Results

Supplementary Video 1. Live cell imaging of A375 cells upon incubation with 20 μM RuBmP. Cell morphology was captured by differential internal reflection fluorescence microscopy (Red: mitochondria; blue: nucleuses; green: RuBmP). Scale bar: 10 μm. Time presented as hr:min:sec.

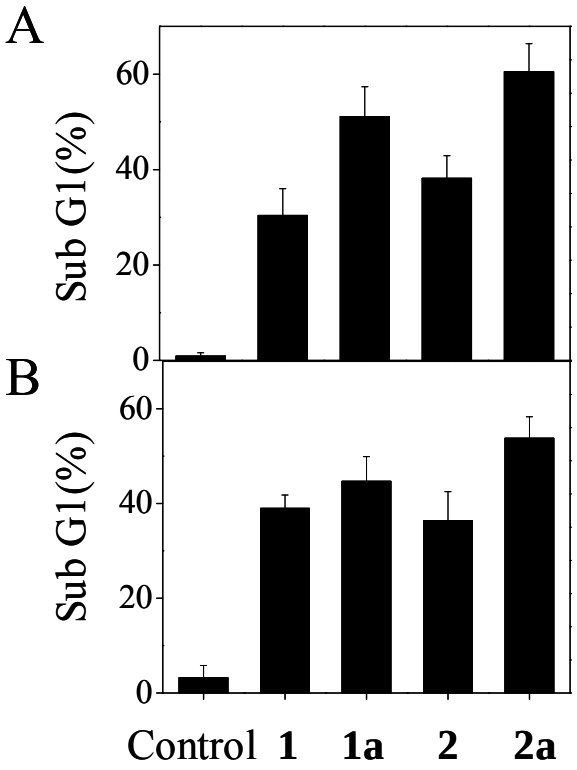

Fig. S1. Apoptotic cell death induced by different Ru complexes as examined by flow cytometric analysis. Cells were treated with Ru complexes for 72 h. (A) A375 cells: **1** (400 μM), **1a** (80 μM), **2** (250 μM), **2a** (40 μM). (B) MCF-7 cells: **1** (100 μM), **1a** (100 μM), **2** (100 μM), **2a** (40 μM).

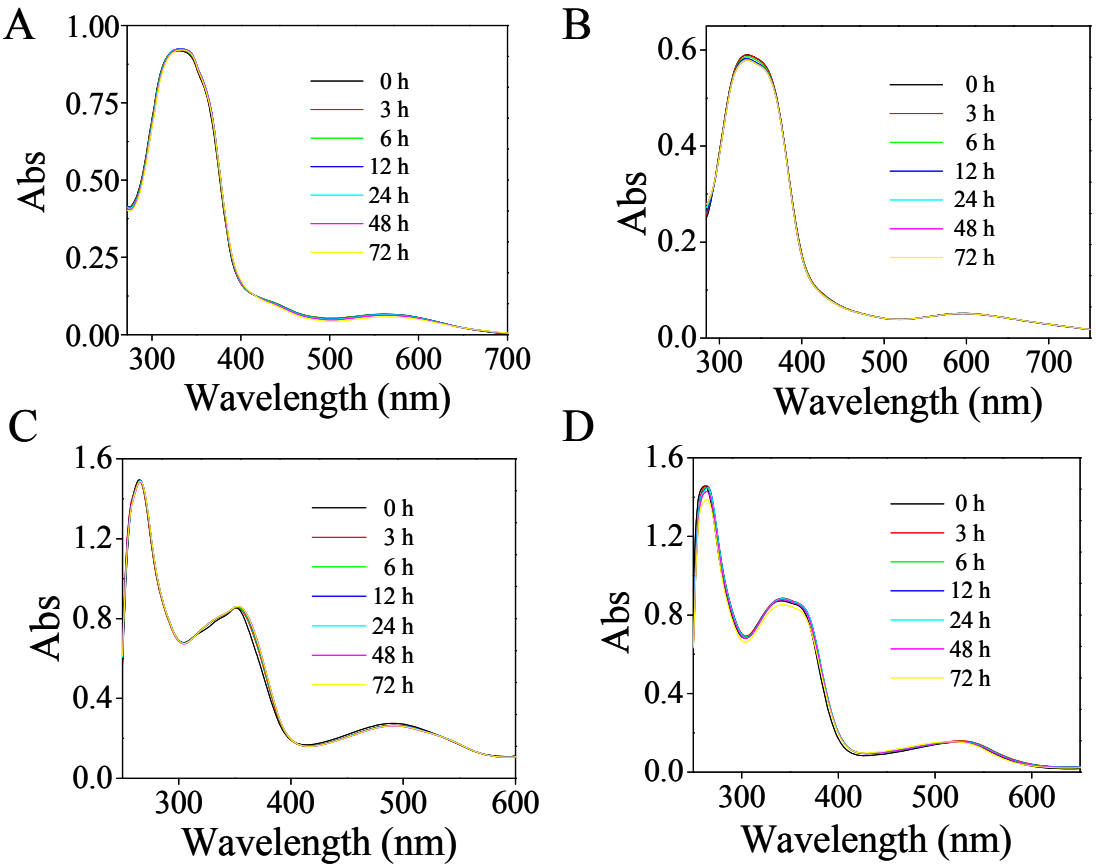

Fig. S2. UV-Vis absorption spectra of complexes **1** (A), **2** (B), **1a** (C) and **2a** (D) in DMSO during incubation at 25℃.

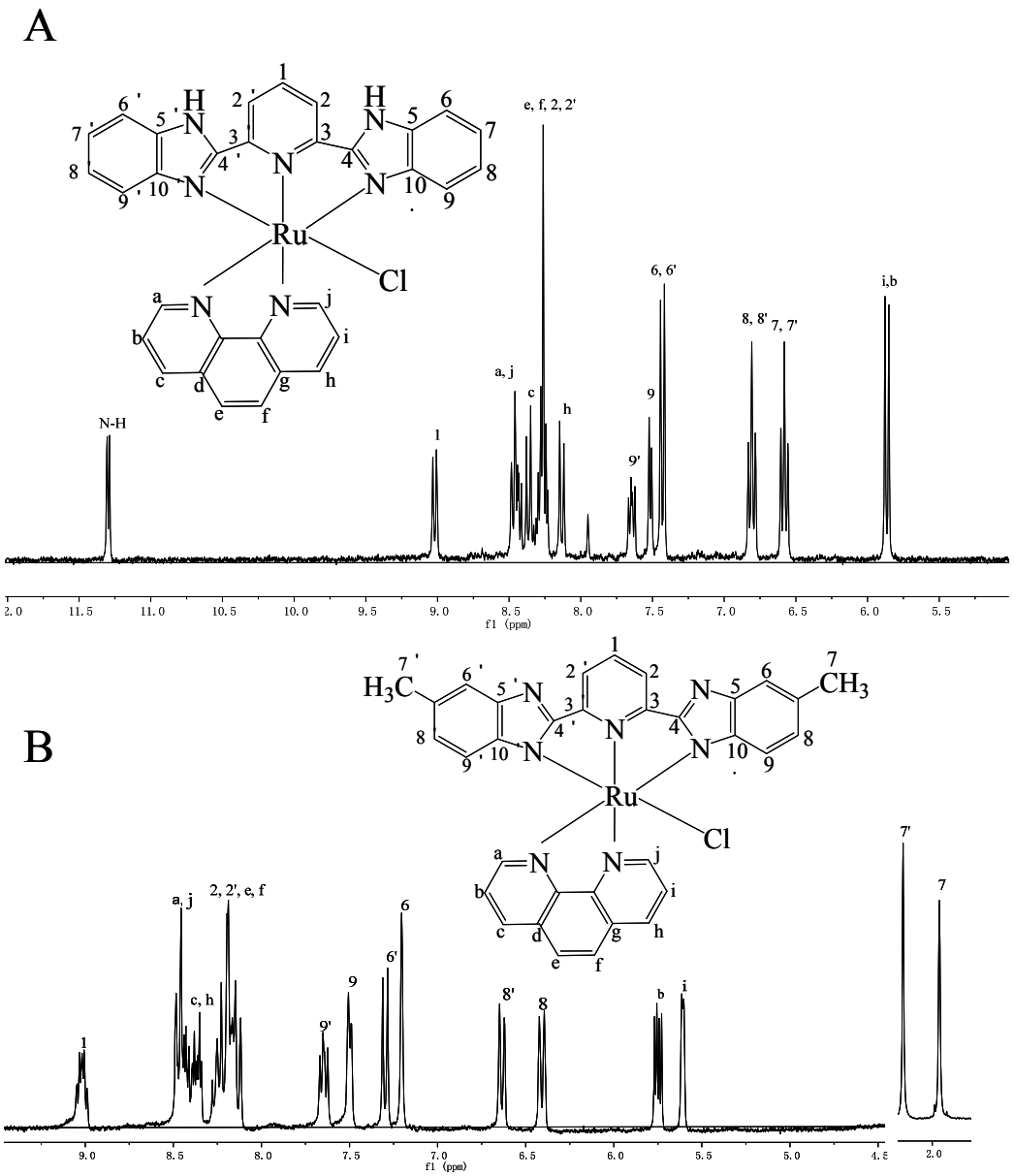

Fig. S3. ^{1}H NMR spectra of complexes **1a** (A) and **2a** (B) (300 MHz, solvent DMSO-d_6).

3. References.

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