Supplementary Material (ESI) for Medicinal Chemistry Communications

Chiral ruthenium polypyridyl complexes as mitochondria-targeted apoptosis inducers

Tianfeng Chen,[a] Wen-Jie Mei, *[a,b] Yum-Shing Wong,[c] Jie Liu,[a] Yanan Liu,[a] Huang-Song Xie[b] and Wen-Jie Zheng*[a]

a Department of Chemistry, Jinan University, Guangzhou 510632, China.

b School of Chemistry and Chemical Engineering, Guangdong Pharmaceutical University, Guangzhou, China.

c Department of Biology, The Chinese University of Hong Kong, Hong Kong SAR, China.

1. Materials and methods

1.1. Materials.

All reagents was obtained commercially and used without further purification unless otherwise noted. Phenanthrolinedione, *cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$, *cis*-$[Ru(bpy)_2(py)_2]Cl_2$, Δ-[Ru-$(bpy)_2(py)_2$] [*o,o′*-dibenzoyltartrate]$\cdot 12H_2O$ and Λ-[Ru-$(bpy)_2(py)_2$][*o,o′*-dibenzoyltartrate]$\cdot 12H_2O$ were prepared and characterized according to the literature[1]. Microanalyses were carried out on an Elementar Vario EL elemental analyzer. ^{1}H NMR spectra were recorded on a Bruker AV-400 MHz spectrometer (Germany) at room temperature and TMS as the internal standard. Electrospray mass spectra (ES-MS) were recorded on a LCQ system (Finnigan MAT, USA). CD spectra were recorded on JASCO J20C spectrophotometer.

1.2. Synthesis and characterization

1.2.1. Synthesis of (4'-trifluoromethylphenyl) imidazo [4,5–*f*][1,10]phenanthroline (*p*-tFMPIP)

The ligand (4'-trifluoromethylphenyl) imidazo [4,5-*f*][1,10] phenanthroline (***p*-tFMPIP**) was prepared by the method modified from the reference[2]. Briefly, a solution of phenanthraquinone (0.652 g, 3 mmol), 4-trifluoromethylphenylaldehyde (0.631 g, 4.5 mmol) and ammonium acetate (4.85 g, 25 mmol) in 10 ml glacial acetic acid was refluxed for 4 hour.

The cooled deep red solution was diluted with 25 ml water, and neutralized with ammonium hydroxide. The mixture was filtered and the precipitates were washed with water and acetone, then dried and purified by chromatography over 60-80 mesh SiO_2 using methanol as an eluent, yield 70.5 %. Calculated for $C_{20}H_{11}F_3N_4 \cdot H_2O$ (%): C, 62.83; H, 3.43; N, 14.65; Found (%): C, 62.64; H, 3.41; N, 14.73. ESI-MS (in DMSO, *m/z*): 365.7 ($[M+H]^+$, calc. 365.1). ^{1}H NMR (in DMSO-d_6, δ/ppm): 9.07 (2H, d); 8.96 (2H, d); 8.52 (2H, d); 8.02 (2H, d); 7.88 (2H, dd).

1.2.2. Synthesis of (3'-trifluoromethylphenyl) imidazo [4,5-*f*][1,10]phenanthroline (*m*-tFMPIP)

(3-trifluoromethylphenyl))imidazo[4,5-*f*][1,10]phenanthroline (***m*-tFMPIP**) was synthesized by the same method as described in section **1.2.1**, but using phenanthraquinone (0.25 g, 1.2 mmol) and 2'-trifluoromethylphenylaldehyde (0.29 g, 1.8 mmol) instead, yield 75.0 %. Calculated for $C_{20}H_{11}F_3N_4 \cdot H_2O$ (%): C, 62.83; H, 3.43; N, 14.65; Found (%): C, 62.32; H, 3.46; N, 14.45. ESI-MS (in DMSO, *m/z*): 365.8 ($[M+H]^+$, calc. 365.1). ^{1}H NMR (in DMSO-d_6,δ/ppm): 8.98 (2H, d); 8.94 (2H, d); 8.65 (1H, s); 8.64 (1H, t); 7.80 (4H, m).

1.2.3. Synthesis of (2'-trifluoromethylphenyl) imidazo [4,5-*f*][1,10]phenanthroline (*o*-tFMPIP)

(2'-trifluoromethylphenyl))imidazo[4,5-*f*][1,10]phenanthroline (***o*-tFMPIP**) was synthesized by the same method as described in section **1.2.1**, but using phenanthraquinone (0.25 g, 1.2 mmol) and 2'-trifluoromethylphenylaldehyde (0.29 g,1.8 mmol) instead, yield 87.0 %. Calculated for $C_{20}H_{11}F_3N_4 \cdot H_2O$ (%): C, 62.83; H, 3.43; N, 14.65; Found (%): C, 62.43; H, 3.48; N, 14.21. ESI-MS (in DMSO, *m/z*): 365.7 ($[M+H]^+$, calc. 365.1). ^{1}H NMR (in DMSO-d_6, δ/ppm): 9.03 (2H, d); 8.88 (2H, d); 7.99 (2H, d); 7.87 (4H, m).

1.2.4. Synthesis of Λ-[Ru(bpy)$_2$(*p*-tFMPIP)]Cl$_2$·3H$_2$O (Λ-1)

Λ-[Ru(bpy)$_2$(*p*-tFPIP)](PF$_6$)$_2$ was synthesized according to the method described in the literature with slight modification[3]. Λ-[Ru(bpy)$_2$(py)$_2$][*o,o'*- dibenzoyltartrate]·12H$_2$O (0.26 g, 0.23 mmol) and *p*-tFMPIP (0.10 g, 0.28 mmol) were added to 20 ml ethylene glycol-water (9:1, v/v). The mixture was refluxed 6 h under an argon atmosphere. The cooled reaction mixture was diluted with water (40 ml) and filtered to remove solid impurities. The complex was then separated from soluble impurities by precipitation with NH_4PF_6. The precipitated complex was dried, dissolved in a small amount of acetonitrile, and purified by

chromatography over alumina oxide using MeCN-toluene (2:1, v/v) as eluent, yield 52.1 %.

The chloride salt was obtained by adding acetone solution saturated by tetra-n-butylammonium chloride. Cacl. for $C_{40}H_{27}Cl_2F_3N_8Ru\cdot 3H_2O$ (%): C, 53.22; H, 3.68; N, 12.41; Found: C, 53.08; H, 3.73; N, 12.27. ESI-MS (in CH_3CN, *m*/*z*): 777.3 ($[M\text{-}3H_2O\text{-}2Cl\text{-}H]^+$, Calc. 777.1), 389.3 ($[M\text{-}3H_2O\text{-}2Cl]^{2+}$, Cacl. 389.1). ^1H NMR (in DMSO-d_6, δ/ppm): 9.10 (2H, m), 8.88 (2H, d), 8.85 (2H, d), 8.54 (2H, d), 8.25 (2H, m), 8.14 (2H, t), 8.12 (2H, d), 8.07 (2H, d), 7.95 (2H, t), 7.88 (2H, d), 7.65 (2H, d), 7.61 (2H, d), 7.37 (2H, t); CD (in Tris-HCl,pH = 7.2, λ_{max} / nm): 291.0 (+).

1.2.5. Synthesis of Δ-[Ru(bpy)$_2$(*p*-tFMPIP)]Cl$_2$·3H$_2$O (Δ-1)

Δ-[Ru(bpy)$_2$(*p*-tFMPIP)](PF$_6$)$_2$ was similarly synthesized but using Δ-Ru(bpy)$_2$-(py)$_2$][*o,o′*-dibenzoyltartrate]·12H$_2$O (0.26 g, 0.23 mmol) and ***p*-tFMPIP** (0.10 g, 0.28 mmol), yield 75.6 %. Cacl. for $C_{40}H_{27}Cl_2F_3N_8Ru\cdot 3H_2O$ (%): C, 53.22; H, 3.68; N, 12.41; Found: C, 53.13; H, 3.45; N, 12.23. ESI-MS (in CH_3CN, *m*/*z*): 777.3 ($[M\text{-}3H_2O\text{-}2Cl\text{-}H]^+$, Cacl.777.1), 389.3 ($[M\text{-}3H_2O\text{-}2Cl]^{2+}$, Cacl. 389.1). CD (in Tris-HCl, pH = 7.2, λ_{max} / nm): 291.0 (-).

1.2.6. Synthesis of Λ-[Ru(bpy)$_2$(*m*-tFMPIP)]Cl$_2$·3H$_2$O (Λ-2)

Λ-[Ru(bpy)$_2$(*m*-tFMPIP)](PF$_6$)$_2$ was similarly synthesized but using ***m*-tFMPIP**(0.10 g, 0.28 mmol) and Λ-Ru(bpy)$_2$(py)$_2$][*o,o′*-dibenzoyltartrate]·12H$_2$O (0.26 g, 0.23 mmol), yield 71.9 %. Cacl. for $C_{40}H_{27}Cl_2F_3N_8Ru\cdot 3H_2O$ (%): C, 53.22; H, 3.68; N, 12.41; Found: C, 52.86; H, 3.83; N, 12.13. ESI-MS (in CH_3CN, *m*/*z*): 777.2 ($[M\text{-}3H_2O\text{-}2Cl\text{-}H]^+$, Calc. 777.1), 389.3 ($[M\text{-}3H_2O\text{-}2Cl]^{2+}$, cacl. 389.1). ^1H NMR (in DMSO-d_6, δ/ppm): 9.09 (2H, d), 8.90 (4H, dd), 8.64 (2H, d), 8.25 (2H, t), 8.14 (2H, t), 8.07 (2H, d), 7.95 (4H, t), 7.87 (2H, d), 7.63 (4H, m), 7.38 (2H, t); CD (in Tris-HCl, pH = 7.2, λ_{max} / nm): 291.0 (+).

1.2.7. Synthesis of Δ-[Ru(bpy)$_2$(*m*-tFMPIP)]Cl$_2$·3H$_2$O (Δ-2)

Δ-[Ru(bpy)$_2$(*m*-tFMPIP)](PF$_6$)$_2$ was similarly synthesized but using ***m*-tFMPIP**(0.10 g, 0.28 mmol) and Δ-Ru(bpy)$_2$(py)$_2$][*o,o′*-dibenzoyltartrate]·12H$_2$O (0.26 g, 0.23 mmol), yield 72.6%. Cacl. for $C_{40}H_{27}Cl_2F_3N_8Ru\cdot 3H_2O$ (%):C 53.22; H 3.68; N 12.41; Found: C, 53.25; H, 3.41; N, 12.32. ESI-MS (in CH_3CN, *m*/*z*): 777.3 ($[M\text{-}3H_2O\text{-}2Cl\text{-}H]^+$, Calc.777.1), 389.3 ($[M\text{-}3H_2O\text{-}2Cl]^{2+}$, Calc. 389.1). CD (in Tris-HCl,pH = 7.2, λ_{max} / nm): 291.0 (-).

1.2.8. Synthesis of Λ-[Ru(bpy)$_2$(*o*-tFMPIP)]Cl$_2$·3H$_2$O (Λ-3)

Λ-[Ru(bpy)$_2$(*o*-tFMPIP)](PF$_6$)$_2$ was similarly synthesized but using ***o*-tFMPIP** (0.10 g, 0.28

mmol) and Λ- Ru(bpy)$_2$(py)$_2$][*o,o′*-dibenzoyltartrate]·12H$_2$O (0.26 g, 0.23 mmol), yield 57.2 %. Cacl. for $C_{40}H_{27}Cl_2F_3N_8Ru·3H_2O$ (%): C, 53.22; H, 3.68; N, 12.41; Found: C, 53.27; H, 3.81; N, 12.50. ESI-MS (in CH_3CN, *m*/*z*) 777.2 ([M-3H$_2$O-2PF$_6$-H]$^+$, Calc. 777.1), 389.3 ([M-3H$_2$O-2PF$_6$]$^{2+}$, Cacl. 389.1). ^{1}H NMR (in DMSO-d$_6$, δ/ppm): 8.92 (6H, m); 8.23 (2H, t); 8.10 (3H, m); 7.88 (2H, d); 7.84 (1H, d); 7.80 (2H, d); 7.75 (3H, m); 7.61 (5H, m); 7.39 (2H, t). CD (in Tris-HCl, pH = 7.2, λ_{max} / nm): 291.0 (+).

1.2.9. Synthesis of Δ-[Ru(bpy)$_2$(*o*-tFMPIP)]Cl$_2$·3H$_2$O (Δ-3)

Δ-[Ru(bpy)$_2$(*o*-tFMPIP)](PF$_6$)$_2$ was similarly synthesized but using ***o*-tFMPIP** (0.10 g, 0.28 mmol) and Δ-Ru(bpy)$_2$(py)$_2$][*o,o′*-dibenzoyltartrate]·12H$_2$O (0.26 g, 0.23 mmol), yield 56.6 %. Cacl. for $C_{40}H_{27}Cl_2F_3N_8Ru·3H_2O$ (%): C, 53.22; H, 3.68; N, 12.41; Found: C, 53.13; H, 3.76; N, 12.32. ESI-MS (in CH_3CN, *m*/*z*) 777.2 ([M-3H$_2$O-2Cl-H]$^+$, Calc.777.1), 389.2 ([M-3H$_2$O-2Cl]$^{2+}$, Calc. 389.1). CD (in Tris-HCl, pH = 7.2, λ_{max} / nm): 291.0 (-).

1.3. Cell lines and cell culture

Human cancer cell lines (melanoma A375, hepatocellular carcinoma HepG2, colorectal adenocarcinoma SW620, and prostate carcinoma PC-3 cells) and human normal cell lines (fibroblast Hs68 and kidney HK-2 cells) were all purchased from American Type Culture Collection (ATCC, Manassas, VA). 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).

1.4. MTT assay

Cell viability was determined by measuring the ability of cells to transform MTT to a purple formazan dye[4]. 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 570 nm using a microplate spectrophotometer (SpectroAmax^{TM} 250).

1.5. Flow cytometric analysis

The cell cycle distribution was analyzed by flow cytometry as previously described[5].

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, pH 8.0) 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.

1.6. 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. 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). To assess the presence of comparable amount of proteins in each lane, the membranes were stripped finally to detect the β-actin.

1.8. 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).

1.9. 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.

2. Results

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

3. References

(1) B.P. Sullivan, D.J. Salmon and T.J. Meyer, *Inorg Chem*, 1978, **17**, 3334-3341.

(2) E.A. Steck, A. R. Day, *J Am Chem Soc,* 1943, **65**, 452-456.

(3) Mei, W.-J.; Wang N.; Liu Y.-J.; Ma Y.-Z.; Wang D.-Y.; Liang B.-X., *Trans Met Chem,* **2008**, *33*, 499–503.

(4) T. Chen and Y.S. Wong, *Biomed Pharmacother*, 2009, **63**, 105-113.

(5) T. Chen and Y.S. Wong, *Int J Biochem Cell Biol,* 2009, **41**, 666-676.

(6) T. Chen and Y.S. Wong, *Cell Mol Life Sci,* 2008, **65**, 2763-2775.