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Ruthenium Polypyridyl Complexes That Induce Mitochondria-Mediated Apoptosis in Cancer Cells

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The limitations of cisplatin-based chemotherapy, including high toxicity, undesirable side effects, and drug resistance, have motivated extensive investigations into alternative metal-based cancer therapies. Ruthenium (Ru) possesses several favorable properties suited to rational anticancer drug design and biological applications. In the present study, we synthesized a series of ruthenium polypyridyl complexes containing N,N-chelating ligands, examined their anticancer activities, and elucidated the molecular mechanisms through which they caused the cancer cell death. The results demonstrated that $[Ru(phen)_2$-*p*-MOPIP$](PF_6)_2 \cdot 2H_2O$ (RuPOP), a complex with potent antiproliferative activity, is able to induce mitochondria-mediated and caspase-dependent apoptosis in human cancer cells. On the basis of these results, we suggest that RuPOP may be a candidate for further evaluation as a chemopreventive and chemotherapeutic agent for human cancers, especially for melanoma.

Cisplatin $[Pt(NH_3)_2Cl_2]$ is one of the most widely used anticancer drugs. Cisplatin-based chemotherapy constitutes a component of standard treatment regimes for testicular, ovarian, cervical, bladder, head and neck, and lung cancers.[1] However, the clinical drawbacks of cisplatin are also apparent, including the limited applicability, the acquired resistance, and the serious side effects, such as neurotoxicity and nephrotoxicity.[2,3] The limitations of cisplatin have motivated extensive investigations into alternative metal-based cancer therapies. Ruthenium (Ru), a rare transition metal of the platinum group, possesses several favorable properties suited to rational anticancer drug design and biological applications, such as ligand-exchange kinetics similar to those of platinum complexes, various oxidation states under physiological conditions, a higher coordination number that could potentially be used to fine-tune the properties of the complexes, and lower toxicity toward healthy tissues by mimicking iron in binding to important carrier proteins.[3,4] A number of Ru complexes have previously been shown to display promising anticancer activities, and two of them, NAMI-A and KP109, have entered clinical trials.[5,6] Several mechanisms have been described to elucidate the anticancer activities of Ru complexes, including inhibition of metastasis,[7] interaction with DNA,[8] production of reactive oxygen species,[9] inhibition of protein kinases,[10] induction of the endoplasmic reticulum stress,[6] and apoptosis.[10] However, the molecular mechanisms and the signaling pathways induced by Ru complexes remain elusive.

Ruthenium polypyridyl complexes comprise a versatile class of compounds with unique electrochemical and photophysical properties that have wide applications as oxidation catalysts, photocatalysts, dye sensitizers for solar cells, fabrication of molecular devices, DNA intercalation, and protein binding.[11−13] Recently, we have synthesized a series of octahedral ruthenium(II) polypyridyl complexes containing N,N-chelating ligands, such as 2,2-bipyridine (bpy) and 1,10-phenanthroline (phen), and investigated their structure−activity relationships in DNA-binding properties and in vitro cytotoxic effects toward human cancer cells.[14,15] The experimental

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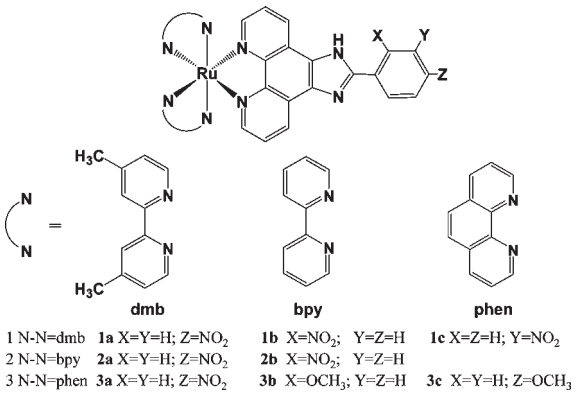

Figure 1. Structures of ruthenium polypyridyl complexes studied in this work.

Table 1. Cytotoxic Effects of Ruthenium Polypyridyl Complexes on Human Cancer and Normal Cell Lines

	IC_{50} (μM)				
complexes	A375	Hep G2	SW620	HS68	HK-2
1a	37.2 ± 1.4	47.3 ± 5.2	46.0 ± 6.2		
1b	78.3 ± 5.8	36.2 ± 2.3	> 200		
1c	36.3 ± 3.7	35.4 ± 4.9	27.3 ± 4.0		
2a	15.1 ± 2.2	10.5 ± 2.4	> 200		
2b	67.8 ± 7.5	52.8 ± 7.1	> 200		
3a	17.4 ± 1.9	10.5 ± 3.0	37.1 ± 4.3		
3b	19.5 ± 3.6	14.5 ± 2.6	58.9 ± 7.7		
3c	5.9 ± 1.1	7.2 ± 1.3	13.6 ± 3.8	33.3 ± 4.5	57.8 ± 6.0
cisplatin	7.3 ± 0.8	13.6 ± 2.0	30.0 ± 4.1	1.8 ± 0.7	10.3 ± 2.1

and theoretical results showed that variation of the ancillary ligands and changes in the positions of substituent groups ($-OCH_3$ and $-NO_2$) on the intercalative ligand could cause interesting difference in the properties of the resulting complexes.[14−16] It was of interest, therefore, in the present work, to examine the anticancer activities of 4,4-dimethyl-2, 2-bipyridine (dmb), bpy, and phen Ru complexes, with substituents $-OCH_3$ and $-NO_2$ at different positions on the phenyl ring (Figure 1), by comparison with cisplatin, and to elucidate the molecular mechanisms through which ruthenium polypyridyl complexes caused the cancer cell death.

Because the balance between the therapeutic potential and toxic side effects of a compound is very important when evaluating its usefulness as a pharmacological drug, experiments were designed to investigate the in vitro cytotoxicity of ruthenium polypyridyl complexes against several human cancer and normal cell lines, including melanoma A375, hepatocellular carcinoma HepG2, colorectal adenocarcinoma SW620, fibroblast Hs68, and HK-2 kidney cells. Table 1 shows the IC_{50} values of eight ruthenium polypyridyl complexes and cisplatin by MTT assay after a 48-h treatment. The tested cancer cells, especially the A375 and HepG2 cells, were susceptible to the complexes. The antiproliferative activities of phen complexes were higher than those of dmb and bpy complexes, as evidenced by the lower IC_{50} values. The most active phen complex, $[Ru(phen)_2$-*o*-MOPIP$](PF_6)_2 \cdot 2H_2O$ (**3c**, RuPOP; PIP = 2-phenylimidazo[4,5-*f*][1,10]phenanthroline), with $-OCH_3$ on the p-site substitution, exhibited a broad spectrum of inhibition on human cancer cells, with

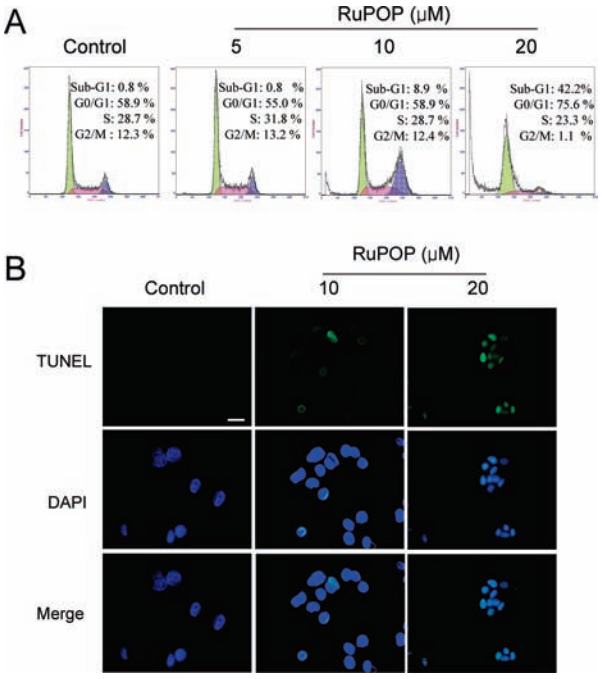

Figure 2. RuPOP-induced apoptotic cell death as examined by flow cytometric analysis (A) and TUNEL assay (B). Cells were treated with different concentrations of RuPOP for 24 h.

IC_{50} values ranging from 5.9 to 13.6 μM, which were lower than those of cisplatin, indicating higher cytotoxic effects of RuPOP on cancer cells. Despite this potency, RuPOP was much less toxic toward human normal cells, with IC_{50} values at 33.3 (Hs68 fibroblasts) and 57.6 μM (HK-2 kidney cells), which are significantly higher than those of cisplatin (4.8 and 3.2 μM). These results suggest that RuPOP possesses great selectivity between cancer and normal cells and displays application potential in cancer chemoprevention and chemotherapy.

Because A375 cells exhibited the highest sensitivity to RuPOP, this cell line was used for further investigation on the underlying mechanisms accounting for the action of RuPOP. First, in vitro DNA-flow cytometric analysis was carried out to determine whether RuPOP-induced cell growth inhibition was the result of apoptosis or cell cycle arrest or a combination of these two modes. The results show that exposure of the A375 cells to different concentrations of RuPOP for 24 h resulted in a marked dose-dependent increase in the proportion of apoptotic cells, as reflected by the subdiploid peak (Figure 2A). Induction of apoptosis by RuPOP was further confirmed by DNA fragmentation and nuclear condensation as examined by TUNEL-DAPI staining assay (Figure 2B). These results indicated that the cell death induced by RuPOP is mainly caused by apoptosis.

Mitochondria act as a point of integration for apoptotic signals originating from both the extrinsic and intrinsic apoptotic pathways.[17,18] Mitochondrial dysfunction and the release of apoptogenic factors are critical events in triggering various apoptotic pathways. Therefore, the status of mitochondria in RuPOP-treated cells was investigated by real-time living cell microscopy. Using MitoTracker Red CMXRos as a marker of mitochondria, we showed that, in

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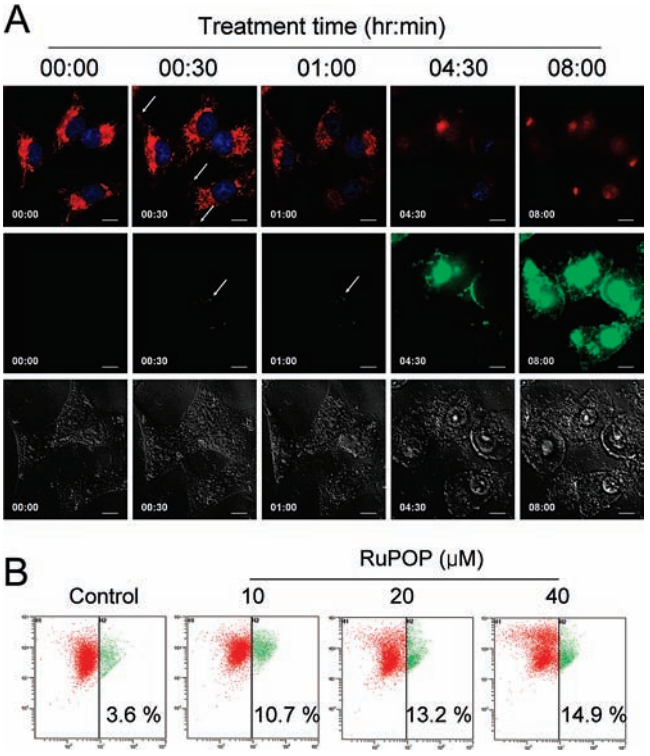

Figure 3. (A) Real-time imaging of the same cells treated with 20 μM RuPOP. The cell morphology was captured by a differential internal reflection (DIC) microscope. Mitochondria, the nucleus, and RuPOP were visualized by red, blue, and green fluorescence, respectively. The upper panel is the merged images of mitochondria and the nucleus. The middle and lower panels are images of RuPOP and DIC, respectively. Scale bar: 10 μm. (B) Loss of $\Delta\Psi_m$ induced by RuPOP. Cells were treated with RuPOP for 3 h and analyzed by JC-1 flow cytometry. The number in each dot plot represents the percentage of cells that lost $\Delta\Psi_m$.

healthy cells, the mitochondrial network was extensively interconnected and appeared filamentous extended throughout the cytoplasm and the nucleus was a round shape. The treatment of RuPOP resulted in mitochondrial fragmentation, the release of mitochondrial contents, nuclear condensation, and cytoplasmic shrinkage. Mitochondrial fragmentation (indicated by the arrows) displayed a rapid onset after 30 min of treatment, followed by a progressive increase to 24 h (Figure 3A and the Supporting Information). Interestingly, we also found that RuPOP emitted green fluorescence in the cells under the living cell microscope, which enables us to examine its cellular uptake easily. As shown in Figure 3A, RuPOP accumulated in the cell membrane after 30 min of treatment (indicated by the arrow) and the cellular RuPOP increased after that. However, we did not observe the overlay of RuPOP fluorescence with nucleus (blue) fluorescence, suggesting that nucleic acids were not the cellular target of RuPOP. Furthermore, the treatment of RuPOP also induced the loss of mitochondrial membrane potential ($\Delta\Psi_m$; Figure 3B), which confirmed activation of mthe itochondria-mediated apoptosis.

Bcl-2 family proteins have been described as key regulators of $\Delta\Psi_m$.[19] In this study, Western blot analysis revealed that RuPOP suppressed the expression of prosurvival Bcl-2 family

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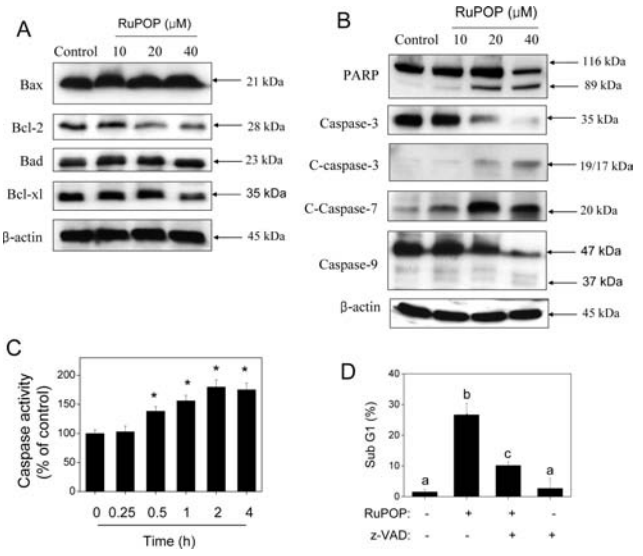

Figure 4. Roles of Bcl-2 and caspase family members in RuPOP-induced apoptosis: (A and B) Cells were treated with RuPOP for 24 h and examined by Western blotting. (C) Cells were treated with 20 μM RuPOP for different times. Asterisks indicate $P < 0.05$ vs controls. (D) The protective effects of z-VAD-fmk on RuPOP-induced apoptosis are shown. Cells were pretreated with z-VAD-fmk for 2 h, followed by coincubation with 20 μM RuPOP for 24 h and flow cytometric analysis. Bars with different characters are statistically different at the $P < 0.05$ level.

proteins, Bcl-2 and Bcl-xl, and upregulated the expression of a proapoptosis Bcl-2 family protein, Bad (Figure 4A). As a result of these changes, the ratios of Bcl-2/Bax and Bcl-xl/Bad decreased significantly, which regulated the loss of $\Delta\Psi_m$ and triggered the mitochondrial release of apoptogenic factors, like cytochrome *c* and AIF. Subsequently, cytosolic cytochrome *c* caused activation of caspase-3, -7, and -9 and cleavage of their specific substrate PARP (Figure 4B). Rapid activation of caspase-9 (30 min) by RuPOP confirmed the early induction of mitochondrial dysfunction (Figure 4C). Furthermore, apoptotic cell death was significantly suppressed by z-VAD-fmk, a general caspase inhibitor, indicating the important roles of caspases in RuPOP-induced apoptosis (Figure 4D).

In conclusion, RuPOP, a potent antiproliferative agent against cancer cells, is able to induce mitochondria-mediated and caspase-dependent apoptosis in human cancer cells. On the basis of these results, we suggest that RuPOP may be a candidate for further evaluation as a chemopreventive and chemotherapeutic agent for human cancers.

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Supporting Information Available: Experimental details for the synthesis and characterization of RuPOP and in vitro cellular studies. This material is available free of charge via the Internet at http://pubs.acs.org.