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# Electronic Effects on Reactivity and Anticancer Activity by Half-Sandwich N, N-Chelated Iridium(III) Complexes

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ABSTRACT: The synthesis and characterization of a series of organometallic half-sandwich N, N-chelated iridium(III) complexes bearing a range of electron-donating and withdrawing substituents were described. The X-ray crystal structures of complexes 1, 3 and 5 have been determined. This work demonstrated how the aqueous chemistry, catalytic activity in converting coenzyme NADH to  $NAD^+$  and anticancer activity can be controlled and fine-tuned by the modification of the ligand electronic perturbations. In general, the introduction of an electron-withdrawing group (-Cl and -NO<sub>2</sub>) on the bipyridine ring resulted in increased anticancer activity, whereas electron-donating group (-NH<sub>2</sub>, -OH and -OCH<sub>3</sub>) decreased anticancer activity. Complex 6 bearing strongly electron-withdrawing NO<sub>2</sub> group displayed the highest anticancer activity  $(7.3 \pm 1.2 \mu M)$ , ca. three times as active as cisplatin in the A549 cell line. Notably, a selective cytotoxicity for cancer cells over normal cells was observed for complexes 1 and 6. DNA binding does not seem to be the primary mechanism for cancer fighting. However, the aqueous chemistry, cell apoptosis and cell cycle, which show the similar dependence on the ligand electronic perturbations as the anticancer activity, appear to together contribute to the anticancer potency of theses complexes. This work may provide an alternative strategy to enhance anticancer activity for these N, N-chelated organometallic half-sandwich iridium(III) complexes.

#### **INTRODUCTION**

In a search for less toxic and more potent alternatives to cisplatin,<sup>1-3</sup> organometallic

complexes have recently been shown to be promising anticancer agents due to their versatile structures, potential redox features, and wide range of ligand substitution rates.<sup>4-18</sup> Among these compounds, half-sandwich iridium(III) complexes bearing bidentate N, N or C, N ligands received great attention for their redox-mediated mechanism of actions (MoAs) different from platinum-based drugs.<sup>19-33</sup>

The Sadler half-sandwich groups have developed two kinds of pentamethylcyclopentadienyl (Cp<sup>\*</sup>) iridium(III) complexes containing neutral N, N-chelated ligands and anionic C, N-chelated ligands respectively (Scheme 1, I and II).<sup>29-31</sup> On the one hand, the biological activity of these complexes increases by the incorporation of phenyl substituents on Cp<sup>\*</sup>. The hydrophobicity and intercalative ability of extended cyclopentadienyl systems make the major contribution to the anticancer activity of their iridium(III) complexes.<sup>31</sup> On the other hand, the electronic and steric properties of the bidentate ligands can also have a large effect on the chemical and biological activities.<sup>30, 32, 33</sup> For example, Sadler et al. have investigated the effects of electron-donating and electron-withdrawing substituents on anionic C, N-chelated ligands on the physiochemical and biological activity of their complexes (II).<sup>30</sup> The anticancer activity also can be fine-tuned by varying the monodentate pyridine derivatives (III). The introduction of an electron-donating group on the pyridine ring resulted in increased anticancer activity.<sup>33</sup> These studies have shown that the anticancer activity of these complexes is strongly dependent on the type of substituent and its position on the ligand, thus providing a new strategy that could be beneficial for the development of these C, N-chelated ligand-based complexes. However, the effect of incorporating functionality on the N. N-chelated ligand in half-sandwich iridium(III) anticancer complexes has not been widely explored. Because of half-sandwich Cp<sup>\*</sup> iridium(III) complexes' wide applications in catalysis,<sup>34-36</sup> previous studies have investigated the electronic effects on the catalytic disproportionation of formic acid to methanol by half-sandwich Cp\* iridium(III) complexes.<sup>36</sup> These results encouraged us to systematically explore the ligand electronic effects on reactivity and cancer cell cytotoxicity of half-sandwich iridium(III) anticancer complexes containing neutral N, N-chelated ligands of 4, 4'-di-R-2, 2'-bipyridine (R = electron-donating and electron-withdrawing groups). In this work, six half-sandwich N, N-chelated iridium(III) complexes bearing a range of electron-donating and withdrawing

substituents (-NH<sub>2</sub>, -OH, -OCH<sub>3</sub>, -H, -Cl or -NO<sub>2</sub>) were synthesized and characterized. These complexes have been investigated for their aqueous chemistry, electrostatic potential surfaces (EPS), nucleobase binding and cancer cell toxicity against A549 cancer cells. The work also explores the MoA of these iridium(III) complexes by catalytic hydride transfer analysis, cell cycle, apoptosis and ROS (reactive oxygen species).

Scheme 1. Half-sandwich Iridium(III) Complexes and Their Substituents Electronic Effect on Anticancer Activity



# **RESULTS AND DISCUSSIONS**

Scheme 2. Synthesis of Iridium(III) Complexes Studied in This Work



**Syntheses.** The synthetic routes for the complexes **1-6** are shown in Scheme 2.  $[Cp^{biph}]$  IrCl<sub>2</sub>]<sub>2</sub> ( $Cp^{biph} = \eta^5 - C_5 Me_4 C_6 H_4 C_6 H_5$ ) were synthesized by microwave heating of IrCl<sub>3</sub>·nH<sub>2</sub>O and relative cyclopentadienyl ligand.<sup>37</sup> These complexes were synthesized in moderate yields by reaction of bipyridine ligands (bpy) with the dinuclear iridium

precursors in methanol at ambient temperature. All complexes were isolated as  $PF_6^-$  salts. The introduction of electron-donating groups (-NH<sub>2</sub>, -OH and -OCH<sub>3</sub>) and electron-withdrawing groups (-Cl and -NO<sub>2</sub>) in complexes **1-3**, **5** and **6** is the first to be reported in complexes of the type [Cp<sup>biph</sup>Ir(N<sup>N</sup>)Cl]PF<sub>6</sub>. All of the synthesized complexes were fully characterized by <sup>1</sup>HNMR (Figure S2-S6 in the Supporting Information), mass spectroscopy (Figure S7-S11 in the Supporting Information), FTIR (Figure S12-S16 in the Supporting Information) and CHN elemental analysis. Single crystals of complexes **1**, **3** and **5** suitable for X-ray diffraction were also obtained by slow evaporation of a methanol/diethyl ether solution at ambient temperature.



Figure 1. X-ray crystal structures with atom numbering schemes for complexes 1, 3 and 5 with thermal ellipsoids drawn at 50% probability. The hydrogen atoms and counteranion  $PF_6^-$  have been omitted for clarity.

**X-ray Crystal Structures.** The molecular structures of  $[Cp^{biph}Ir(bpy-NH_2)Cl]PF_6$  (1),  $[Cp^{biph}Ir(bpy-OCH_3)Cl]PF_6$  (3) and  $[Cp^{biph}Ir(bpy-Cl)Cl]PF_6$  (5) were determined by X-ray diffraction and are shown in Figure 1. CCDC numbers of 1, 3 and 5 are 1569686, 1569665 and 1569685, respectively. The selected bond lengths and angles are shown in Table 1 and crystallographic data are listed in Table S1. Complexes 1, 3 and 5 exhibit the expected half-sandwich pseudo-octahedral "three-leg piano-stool" geometry, similar to reported complex 4.<sup>29</sup> The distances of iridium bound to  $\eta^5$ -cyclopentadienyl ring centroid for complexes 1, 3 and 5 are 1.781, 1.814 and 1.795 Å, respectively. The complex 1 has the longest Ir-Cl bond length of these three X-ray structures. The Ir-N<sub>1</sub> (chelating ligand) bond lengths in 1, 3 and 5 are 2.098 (5), 2.095 (8) and 2.115 (5) Å, respectively and the Ir-N<sub>2</sub> bond lengths are 2.103 (5), 2.082 (8) and 2.095 (5) Å,

electron-withdrawing groups on the bipyridine can have a certain degree of influence on the structure.

	1	3	5
Ir-C(cyclopentadienyl)	2.147(6)	2.189(12)	2.155(6)
	2.152(6)	2.187(11)	2.169(6)
	2.159(7)	2.177(10)	2.170(6)
	2.173(6)	2.184(10)	2.178(7)
	2.186(7)	2.179(11)	2.201(7)
Ir-C(centroid)	1.7813	1.8135	1.7948
Ir–N <sub>1</sub>	2.098(5)	2.095(8)	2.115(5)
Ir-N <sub>2</sub>	2.103(5)	2.082(8)	2.095(5)
Ir-Cl	2.4103(18)	2.244(10)	2.3856(18)
N <sub>1</sub> -Ir-N <sub>2</sub>	76.62(19)	76.7(3)	76.1(2)
N <sub>1</sub> -Ir-Cl	84.77(16)	83.0(3)	85.65(15)
N <sub>2</sub> -Ir-Cl	84.54(15)	84.4(4)	87.30(16)

Table 1. Selected Bond Lengths (Å) and Angles (deg) for Complexes 1, 3 and 5.

Electrostatic potential surfaces. The electrostatic potentials surfaces (EPSs) of complexes 1-6 were calculated performing in the framework of density functional theory (DFT) by using of the hybrid B3LYP function implemented in the Gaussian 09 program package.<sup>38</sup> We select the Los Alamos LANL2DZ effective nuclear pseudopotentials (ECPs) and valence bis-zeta group for the iridium atom and the 6-31+G (d, p) basis group for the carbon, nitrogen, chlorine, oxygen and hydrogen atoms.<sup>39,40</sup> The structures of the complexes were fully optimized without any symmetry constraints. Charge delocalization was performed using natural bond orbital (NBO) analysis.<sup>41</sup> The resulting EPS of each complex is shown in Figure 2. There are no major differences in the charge distribution at the iridium center, Cp<sup>biph</sup> ring and chloride ligands among the complexes 1-6. The electron-donating and electron-withdrawing substituent on the chelating ligand only causes a localized effect. The EPS of the chelating ligand are affected by enhancing or diminishing the electronic charge density on the bipyridine ring to which the substituent is bound. Complexes 5 and 6 bearing the electron-withdrawing groups provide a more positive EPS within the ring, while complexes 1-3 containing the electron-donating groups generate a more negative EPS within the ring. Weak electrostatic forces are often

important for binding and recognition interactions with biomolecules such as peptides, proteins, and enzymes.<sup>42</sup> As a result, the nature of the substituent may play a key role in the interaction of the complexes with target sites.



**Figure 2.** EPSs of the complexes **1-6**. EPSs surfaces are shown in both space (with positive and negative regions in blue and red, respectively) and mapped on electron density (isovalue 0.004) of the molecules. The electrostatic potential is represented with a color scale going from red (-0.15 au) to blue (+0.15 au).

Scheme 3. Behaviour of Complexes 1-6 in Methanol/Water Solutions.



**Hydrolysis Studies.** Since M-OH<sub>2</sub> complexes often show higher activity than the corresponding chlorido complexes.<sup>43, 44</sup> Hydrolysis of M-Cl bonds is considered to be an activation step for anticancer complexes.<sup>45</sup> Due to limited solubility in water, the hydrolysis studies on complexes **1-6** were carried out in 50% CD<sub>3</sub>OD /50% D<sub>2</sub>O (v/v) solutions by <sup>1</sup>HNMR at 298 K. No hydrolysis was observed by <sup>1</sup>HNMR spectroscopy. In addition, no obvious changes in the <sup>1</sup>HNMR spectra of complexes **1-6** can be observed

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over 24 h. It is possible that the agua complex was obtained before the <sup>1</sup>HNMR spectra were acquired. Therefore, NaCl (20 mol equiv) was then added to the solutions to further confirm the hydrolysis of these complexes. There was also no change in the <sup>1</sup>HNMR spectra (Figure S17-S23 in the Supporting Information). In addition, when AgNO<sub>3</sub> (0.95 mol equiv) was added to the same solution of complex  $\mathbf{3}$ , the peaks of agua complex was observed after 10 h. These results indicated that the complexes were stable towards hydrolysis under these conditions (Figure S20 in the Supporting Information). Previous studies have shown that the hydrolysis of the similar complexes can be detected by UV-Vis spectroscopy.<sup>29</sup> As a result, the hydrolysis of complexes **1-6** in 10% MeOH/90% H<sub>2</sub>O (v/v) solution was also monitored by ESI-MS and UV-Vis (Figure S24-S35 in the Supporting Information). As shown in Scheme 3, metal species [X-H<sub>2</sub>O]<sup>2+</sup> or [X-H<sub>2</sub>O-H]  $^{+}(X = Cp^{biph}Ir(N^N)H_2O)$ , which arise from the Cl<sup>-</sup>/H<sub>2</sub>O exchange, were detected by ESI-MS. The aqua adducts  $[Cp^{biph}Ir(N^N)H_2O]^{2+}$  may lose a H<sub>2</sub>O or H<sub>3</sub>O<sup>+</sup> in the testing process.<sup>46</sup> Furthermore, the NMR analysis mentioned above showed that the complexes in water-methanol solution were still Cp<sup>biph</sup>- and bpy-bound Ir(III) species and did not suffer from decomposition or ligand dissociation. These results suggested that the hydrolysis of complexes 1-6 occurred in 10% MeOH/90% H<sub>2</sub>O (v/v) solution. Thus, the absorbance changes in UV-Vis spectra can be ascribed to the production of aqua adducts. However, when 50% MeOH/50% H<sub>2</sub>O (v/v) solution was employed, no absorbance changes were observed in UV-Vis spectra (Figures S24(B)-S29(B) in the Supporting Information), which indicated that no hydrolysis occurred under these conditions. This result was consistent with NMR analysis. It seems reasonable that, in accordance with the behavior of some previously reported half-sandwich metal complexes.<sup>47-49</sup> complexes 1-6 may undergo Cl<sup>-</sup>/H<sub>2</sub>O exchange more easily in diluted solutions with a higher relative content of water, as is the case of cell culture. Hydrolysis rate constants and half-lives can be calculated (Table 2) by fitting the UV-Vis absorption difference to pseudo first-order kinetics. To a certain extent, the hydrolysis of Ir-Cl bonds is influenced by the electronic perturbation. Basically, the half-lives of hydrolysis were observed to be lower for the complexes with electron-withdrawing substituents. Complex 3 containing the electron-donating OCH<sub>3</sub> group showed the longest half-life (78.0  $\pm$  6.0 min, Table 2), which is almost ca. 13 times as that of complex 6 containing the electron-withdrawing

 $NO_2$  group. This result may attribute to the shortest Ir-Cl bond of complex 3 (2.244 Å), which facilitate chloride loss.

Complex	$k (min^{-1})$	$t_{1/2}(\min)$
1	0.023	$31.1 \pm 3.2$
2	0.013	$54.0 \pm 1.0$
3	0.009	$78.0 \pm 6.0$
4	0.010	$70.2 \pm 5.6$
5	0.024	$29.0 \pm 2.8$
6	0.117	$6.0 \pm 0.8$

**Table 2.** Hydrolysis Data for Complexes 1-6 at 298K Monitored by UV-Vis.

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Cytotoxicity. The cytotoxicity of complexes 1-6 in A549 human lung cancer cells was shown in the Table 3. The  $IC_{50}$  value (concentration at which 50% of the cell growth is inhibited) covered a wide range from 7.3 to 35.2  $\mu$ M in A549 cells, indicating that the electronic perturbations exhibit some variation on the cytotoxicity of the complexes. The  $IC_{50}$  value revealed the following trends in activity:  $NO_2 > Cl > H > OCH_3 > NH_2 > OH$ . It is worth noting that the presence of electron-withdrawing groups in complexes increases the cytotoxicity, which is opposite to the reported C, N-chelated iridium complexes<sup>33</sup> (Scheme 1, III) with a substituent on the monodentate pyridine ring. Complex 6 bearing electron-withdrawing  $NO_2$  group displays the most potent anticancer activity  $(7.3 \pm 1.2 \ \mu\text{M})$  compared to other complexes 1-5. In particular, IC<sub>50</sub> values of complex 6 are ca. three times as active as cisplatin in the A549 cell line  $(7.3 \pm 1.2 \mu M V s)$  $21.3 \pm 1.7 \mu$ M), demonstrating great potential in cancer chemotherapy. Previously studies have shown that iridium bipydine-chelated half sandwich complexes can hydrolyze rapidly and may have a major contribution to anticancer activity.<sup>29</sup> In our system, although the structure activity relationships are not very significant, the complexes containing the electron-withdrawing groups seem to have the faster hydrolysis rate and the higher anticancer activity than that containing the electron-donating groups, indicating that activation by aquation is crucial to the MoA of these iridium(III) complexes.

The cytotoxicity of complexes **1-6** were further studied against two human bronchial epithelial normal cells BEAS-2B and 16HBE, in the Table 3. Interestingly, complexes **1** and **6** both exhibited much less cytotoxicity towards normal cells than cancer cells. Notably, complex **6** achieved good selectivity with  $IC_{50}$  ratio of 3.0 and 5.9 of BEAS-2B

and 16HBE normal cells to A549 cancer cells. However, complexes **1** and **6** are still active against human bronchial epithelial cells BEAS-2B and 16HBE normal cells with  $IC_{50}$ < 75  $\mu$ M. Hence, more structural modification is necessary to decrease the cytotoxic action towards normal cells without loss the selectivity between cancer and normal cells in the future work.

**Table 3**. Inhibition of Growth of A549 Human Lung Cancer Cells by Complexes **1–6** and Comparison with Cisplatin; Complexes **1**, **4**, **6** and Cisplatin Comparison with BEAS-2B and 16HBE.<sup>a</sup>

Complex	IC <sub>50</sub> (μM) A549	IC <sub>50</sub> (µM) BEAS-2B	IC <sub>50</sub> (μM) 16HBE	$R^1$	R <sup>2</sup>
1	$30.7 \pm 1.4$	$70.3 \pm 1.9$	$74.6\pm0.6$	2.3	2.4
2	$35.2 \pm 1.4$	$28.1 \pm 3.1$	$19.6 \pm 1.4$	0.7	0.5
3	$14.9\pm0.3$	$14.7 \pm 1.3$	$26.6 \pm 1.2$	0.9	1.8
4	$14.3 \pm 1.2$	$14.3 \pm 1.3$	$16.7 \pm 1.7$	1.0	1.2
5	$11.3 \pm 0.9$	$5.0 \pm 0.4$	$3.9 \pm 0.1$	0.4	0.3
6	$7.3 \pm 1.2$	$21.7 \pm 2.3$	$43.0 \pm 3.7$	3.0	5.9
Cisplatin	$21.3 \pm 1.7$	$42.0\pm2.3$	$18.6 \pm 2.0$	2.0	0.9

<sup>a</sup> Cells were treated with various concentrations of tested compounds for 24 h. Cell viability was determined by the MTT assay and  $IC_{50}$  values were calculated as described in the Experimental Section. Each value represents the mean SD of three independent experiments. R<sup>1</sup> represents the ratio of  $IC_{50}$  of BEAS-2B normal cells to A549 cancer cells. R<sup>2</sup> represents the ratio of  $IC_{50}$  of 16HBE normal cells to A549 cancer cells.

**Interaction with Nucleobases.** DNA often represents a potential target site for many transition metal anticancer complexes,<sup>50, 51</sup> complexes **1-6** were evaluated for their ability to bind to 9-ethylguanine (9-EtG) and 9-Methyladenine (9-MeA). 9-MeA or 9-EtG was added to an equilibrium solution of complexes **1-6** (1.0 mM) in 50% CD<sub>3</sub>OD /50% D<sub>2</sub>O (v/v) at 310 K. No new peaks were detected in the <sup>1</sup>HNMR over a period of 24 h (Figure S36-S38 in the Supporting Information), indicating that complexes **1-6** did not show any binding to model nucleobases 9-MeA under these conditions (Table S3 in the Supporting Information). Also, the formation of nucleobase adducts by these iridium(III) complexes was not detected by mass spectrometry. So DNA may not be the main target for this type of iridium(III) complexes and the regulation of the electronic effect also does not affect

Cleavage plasmid DNA. We also studied the DNA binding ability of complexes 1, 6 through agarose gel electrophoresis of supercoiled pBR322 plasmid DNA in buffer. However, no DNA cleavage can be observed for these iridium(III) complexes even at a high concentration of 150  $\mu$ M. Likewise, DNA may not be the major target of these complexes. (Figure S39-S40 in the Supporting Information).

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**Reaction with NADH.** Coenzyme NADH and NAD<sup>+</sup> plays a crucial role in a series of biocatalyzed processes. Previously, we have demonstrated that NADH can donate a hydride to aqua C, N-chelated iridium(III) complexes and produce ROS in the form of H<sub>2</sub>O<sub>2</sub>, thus providing a catalytic behavior to an oxidant mechanism of action.<sup>52, 53</sup> As a result, reactions between iridium(III) complexes **1-6** and NADH were studied (Figure 3). The reaction of a 0.9 mM solution of complex 1 with NADH (3.6 mol equiv.) in 50% CD<sub>3</sub>OD /50% D<sub>2</sub>O (v/v) at 298 K was monitored by <sup>1</sup>HNMR spectroscopy over a 7 h period. After 2 h, a sharp singlet peak at -10.91 ppm, which is corresponding to Ir-H species, was observed. After 7 h, this peak was still visible. A new set of peaks in the <sup>1</sup>H NMR spectra corresponding to the formation of NAD<sup>+</sup> was also observed. In addition, the reaction of complexes 1-6 with NADH in 1: (ca. 90) ratio in 10% MeOH/90% H<sub>2</sub>O (v/v) was also monitored by UV-Vis at 298 K in order to examine catalytic ability (Figure S41 in the Supporting Information). The conversion of NADH to NAD<sup>+</sup> can be measured by the amount of UV absorption at 339 nm as NADH has an absorption at 339 nm while  $NAD^+$  not. The turnover numbers (TONs) of complexes calculated by the amount of UV absorption at 339 nm (Figure 3) revealed the following trends in catalytic activity: 1 (35.3), **2** (1.4), **3** (4.4), **4** (1.0), **5** (5.3) and **6** (36.3), NO<sub>2</sub> > NH<sub>2</sub> > Cl > OCH<sub>3</sub> > OH > H. Despite no clear trend was observed, it is apparent that ligand electronic effects were pronounced. Complex  $\mathbf{6}$  exhibits the best catalytic activity, which is 8 times as much as that of complex 3. The presence of  $NH_2$  and  $NO_2$  group in complexes 1 and 6 seems to enhance the catalytic activity. Very good catalytic activity of the oxidation of NADH was achieved, with TONs 35.3 for complex 1 and 36.3 for complex 6. As these complexes can covert NADH to NAD<sup>+</sup>, the good catalytic performance may provide a potential pathway

to induce of ROS and enhance the killing of cancer cells in an oxidant mechanism of action.<sup>52</sup> In a solution of **6** (0.5 mM) with NADH (3 mol equiv) in 50% MeOH/ 50%H<sub>2</sub>O (v/v) at 298 K, the ROS hydrogen peroxide was detected by the appearance of a blue color on hydrogen peroxide-test paper. It is found that the concentration of H<sub>2</sub>O<sub>2</sub> is about 0.20 mM, and its level may be limited by the oxygen solubility (ca. 0.23 mM at 288 K).<sup>52</sup> However, complexes **1** and **6**, which had the opposite electronic contribution, behaved the similar catalytic activity toward NADH reaction. This result suggested the formation of Ir-H did not play a vital role in killing cancer cells in this system. It is expected that the anticancer activity of these complexes is more dependent on other pathways such as the cell cycle arrest.



**Figure 3.** (A) <sup>1</sup>HNMR spectra of the reaction between complex [Cp<sup>biph</sup> Ir(bpy-NH<sub>2</sub>)Cl]PF<sub>6</sub> (1) (0.9 mM) and NADH (3.6 mol equiv.) in 50% CD<sub>3</sub>OD /50% D<sub>2</sub>O (v/v) at 298 K, 5 min; 2.0 h; 7.0 h. Left: low-field region; right: high-field region showing the Ir-H hydride peak (-10.91 ppm). (B) The normalized UV-Vis spectra of the reaction of NADH (ca. 90  $\mu$ M) with complex [Cp<sup>biph</sup>Ir(bpy-NH<sub>2</sub>)Cl]PF<sub>6</sub> (1), [Cp<sup>biph</sup> Ir (bpy-NO<sub>2</sub>)-Cl]PF<sub>6</sub> (6) (1  $\mu$ M) respectively in 10% MeOH / 90% H<sub>2</sub>O (v/v) at 298 K for 8 h. (C) The turnover numbers (TONs) of complexes 1-6.

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**Apoptosis Assay.** Complex 1 containing electron-donating  $NH_2$  group and complex 6 containing electron-withdrawing  $NO_2$  group were selected for more investigation on their mechanism of actions. A lot of metal-based anticancer drugs have been reported to inhibit cell growth by inducing apoptosis, which is regard as a programmed cell death process.<sup>54-62</sup> Hence, complexes 1 and 6 at 0.5, 1 and 2 equipotent concentrations of  $IC_{50}$ were used for the treatment of A549 human lung cancer cells for 24 h, which were then stained with Annexin V/Propidium Iodide and analyzed by flow cytometry. Cell populations as viable, early apoptosis, late apoptosis and nonviable can be determined through this experiment. The results were shown in Figure 4 and Table S4 in the supporting information. In general, cells in apoptosis increased with the concentrations of iridium(III) complexes. The plots showed that around 16.8% A549 cells were in early apoptotic phase and 28.9% cells were in the late apoptotic phase after 24 h of exposure to complex 1 at a concentration of IC<sub>50</sub>. When the complex 1 is at  $2 \times IC_{50}$  concentrations, 52.3% A549 cells were in apoptosis phase (the total proportion of early and late apoptotic cells), whereas untreated cells remained 90.4% viable, indicating obvious induction of apoptosis at  $2 \times IC_{50}$  concentrations. Complex 6 showed the similar mode of action. The proportion of early and late apoptotic cells was 22.9% and 37.6%, respectively at a concentration of IC<sub>50</sub> after 24 h. At 2  $\times$  IC<sub>50</sub> concentrations, a total of 83.1% (the total proportion of early and late apoptotic cells) cells were undergoing apoptosis. Therefore, the complex 6 containing the electron-withdrawing  $NO_2$  groups has the stronger ability to induce apoptosis of A549 cells, which is consistent with its higher anticancer activity compared to complex 1 containing the electron-donating  $NH_2$  groups.

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**Figure 4.** Apoptosis analysis of A549 cells after 24 h of exposure to complexes **1** and **6** at 310 K determined by flow cytometry using Annexin V-FITC vs PI staining. (A) A549

cells were control and treated with different concentrations of complexes 1 and 6 for 24 h; (B) Populations for cells treated by complexes 1 and 6.

**Cell Cycle Analysis.** Through the cell cycle analysis, we can determine whether the induced cell growth inhibition was the result of cell cycle arrest.<sup>63</sup> Hence, cell cycle arrest analysis for complexes **1** and **6** toward A549 human lung cancer cells was performed by flow cytometry (Table 5 and Table S5-S6 in the Supporting Information). Treating of A549 cells with complex **1** at a concentration of  $0.25 \times IC_{50}$  and  $0.5 \times IC_{50}$  led to G<sub>1</sub> phase arrest, where the percentages of cells increased 1.5% and 5.9% respectively compared to untreated cells. This small change was deemed as a very weak cell cycle arrest ability and showed significant increases in the proportions of cells at G<sub>2</sub>/M phase, about 5.9% and 10.8% respectively at a concentration of  $0.25 \times IC_{50}$  and  $0.5 \times IC_{50}$ . In addition, a small sub-diploid peak corresponds to sub-G<sub>1</sub> phase change was observed for complex **6**, indicating that cells are undergoing apoptosis. In consistent with apoptosis assay analysis, introducing electron-withdrawing groups to the complexes can enhance the cell cycle arrest.



Figure 5. Cell cycle analysis of A549 cells after 24 h of exposure to complexes 1 and 6 at 310 K. Concentrations used were 0.25 and 0.5 equipotent concentrations of  $IC_{50}$ . Cell staining for flow cytometry was carried out using PI/RNase. (A) FL2 histogram for negative control (cells untreated) and complexes 1 and 6. (B) Cell populations in each cell cycle phase for control and complexes 1 and 6.

Induction of ROS in A549 Human Lung Cancer Cells. High concentration of

reactive oxygen species (ROS) often result in oxidative stress and damages to cells.<sup>64</sup> The level of reactive oxygen species (ROS) in A549 human lung cancer cells induced by complexes **1** and **6** at concentrations of  $0.25 \times IC_{50}$  and  $0.5 \times IC_{50}$  are also determined by flow cytometry analysis (Figure 6 and Table S7-S8 in the Supporting Information). Compared to untreated cells, a dramatic increase in the ROS levels was observed for complexes **1** and **6** even at low concentration ( $0.25 \times IC_{50}$ ) after 24 h of drug exposure. The generation of ROS by the complexes may provide a basis for killing cancer cells. It should be noted that the increased ROS levels in cancer cells by these iridium(III) complexes may be attributed to the catalytic reaction of NADH to NAD<sup>+</sup>. On the one hand, the possible chemical mechanism of induction of ROS induced by iridium(III) complexes has been reported previously.<sup>52</sup> On the other hand, complexes **1** and **6**, which displayed the similar catalytic activity toward the reaction of NADH to NAD<sup>+</sup>, also generated the similar amounts of ROS. However, considering the different anticancer activity of complexes **1** and **6**, producing ROS also seems to not be a major contributor to the cytotoxicity in this system.



**Figure 6.** Flow cytometry analysis on ROS induction in A549 human lung cancer cells treated with complex **1** and **6**.

## CONCLUSION

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In summary, a series of N, N-chelated iridium complexes bearing a series of

electron-donating and -withdrawing substituents were synthesized. The aim is to systematically investigate the ligand electronic effects on reactivity and cancer cell cytotoxicity in this class of half-sandwich iridium(III) complexes. The X-ray crystal structures of complexes 1, 3 and 5 were obtained. DFT calculations showed that substituents caused only localized effects on the electrostatic potential surface of the complexes. The more rapid hydrolysis rate of Ir-Cl bonds was obtained with complexes bearing more strongly electron-withdrawing groups. No nucleobase binding was observed for this type of complexes under the conditions mentioned in the experiment. All complexes showed promising activity toward the A549 human lung cancer cell, comparable to, and for some complexes even higher than the clinical anticancer drug cisplatin. The ligand electronic structure of the complexes had some influence on the anticancer activity. Complex 6 bearing strongly electron-withdrawing  $NO_2$  group displayed the highest anticancer activity, ca. three times as active as cisplatin in the A549 cell line, but even more importantly, complexes 1 and 6 displayed the anticancer selectivity. The work also explores the mechanism of actions (MoAs) of these iridium(III) complexes by cell apoptosis and cycle. Both two experiments also exhibited the same trend that the electron-withdrawing groups on the bipyriding ring led to better effect than the electron-donating groups. In addition, these iridium complexes can also induce a dramatic increase in the level of ROS in A549 cancer cells.

Pervious work has shown that the biological activity of these complexes increases by the incorporation of phenyl substituents on Cp<sup>\*</sup>.<sup>29, 31</sup> This study shows that the electronic changes in the bipyridine in half-sandwich iridium complexes can also have an effect on the chemical and biological activity of these complexes. This may provide an alternative strategy to enhance anticancer activity for these N, N-chelated organometallic half-sandwich iridium anticancer complexes.

### **EXPERIMENTAL SECTION**

**Materials**. IrCl<sub>3</sub>·nH<sub>2</sub>O, 2,3,4,5-tetramethyl-2-cyclopentenone (95%), 4-bromo-biphenyl, butyllithium solution (1.6 M in hexane), 4, 4'-dimethoxy-2, 2'-bipyridine, 2, 2'-bipyridine, concentrated sulfuric acid, fuming nitric acid, 10% Pd/C, hydrazine hydrate, ammonium hexafluorophosphate and NaCl, anhydrous MgSO<sub>4</sub>, HCl (36%), HBr solution (41 %), hexane, diethyl ether, ethanol, dry THF, 9-ethylguanine and 9-methyladenine were purchased from Sigma-Aldrich. Cp<sup>xbiph</sup>H, [Cp<sup>biph</sup>IrCl<sub>2</sub>]<sub>2</sub> and complex **4** were synthesized according to the reported methods.<sup>29, 37</sup> For the biological experiments, phosphate-buffered saline (PBS), penicillin/streptomycin mixture, fetal bovine serum, trypsin/EDTA and DMEM medium were purchased from Sangon Biotech.. Testing compounds was dissolved in DMSO and diluted with the tissue culture medium before use.

[Cp<sup>biph</sup>Ir(bpy-NH<sub>2</sub>)Cl]PF<sub>6</sub> (1). A solution of  $[Cp^{biph}IrCl_2]_2$  (108 mg, 0.1 mmol) and 4, 4'-diamino-2, 2'-bipyridine (47.4 mg, 0.25 mmol) in MeOH was heated to reflux in an N<sub>2</sub> atmosphere for 16 hours, cooled and filtered. The volume of the solution was slowly reduced to a small amount on a rotary evaporator, NH<sub>4</sub>PF<sub>6</sub> (135 mg, 0.8 mmol) was added and after stirring at 277 K for 0.5 h, a precipitate formed in the solution. It was collected by filtration, washed with ether and recrystallized from methanol/ether. Yield: 52 mg (32%). A single crystal was obtained by slow evaporation of a methanol/diethyl ether solution at room temperature. <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>):  $\delta$  8.00 (d, J = 6.6 Hz, Ar-*H*, 2H), 7.75 (t, J = 7.1 Hz, Ar-*H*, 4H), 7.53-7.47 (m, Ar-*H*, 4H), 7.41 (t, J = 7.4 Hz, Ar-*H*, 1H), 7.27 (s, NH<sub>2</sub>, 4H), 7.23 (d, J = 2.5 Hz, Ar-*H*, 2H), 6.71 (dd, J = 6.6, 2.5 Hz, Ar-*H*, 2H), 1.73 (s, Cp<sup>biph</sup>-*H*, 6H), 1.66 (s, Cp<sup>biph</sup>-*H*, 6H). Anal. Calcd for [Cp<sup>biph</sup>Ir(bpy-NH<sub>2</sub>)Cl]PF<sub>6</sub> (832.25): C, 44.74; H, 3.75; N, 6.73. Found: C, 44.62; H, 3.71; N, 6.76. MS: m/z 687.02 [Cp<sup>biph</sup>Ir(bpy-NH<sub>2</sub>)Cl]<sup>+</sup>. IR (KBr pellets, cm<sup>-1</sup>): 3461 (NH<sub>2</sub>), 3361 (NH), 1640 (NH), 1318 (C-N).

[Cp<sup>biph</sup>Ir(bpy-OH)Cl]PF<sub>6</sub> (2). The synthesis was performed as for 1 using  $[Cp^{biph}IrCl_2]_2$  (72 mg, 0.07 mmol) and 4, 4'-dihydroxy-2, 2'-bipyridine (30.7 mg, 0.16 mmol). Yield: 63 mg (56%). <sup>1</sup>HNMR (500 MHz, DMSO-d<sub>6</sub>):  $\delta$  7.99 (s, OH, 2H), 7.74 (s, Ar-*H*, 4H), 7.53 (d, J = 8.0 Hz, Ar-*H*, 3H), 7.48 (d, J = 7.2 Hz, Ar-*H*, 2H), 7.40 (s, Ar-*H*, 2H), 7.28 (s, Ar-*H*, 2H), 6.60 (s, Ar-*H*, 2H), 1.71 (s, Cp<sup>biph</sup>-*H*, 6H), 1.64 (s, Cp<sup>biph</sup>-*H*, 6H). Anal. Calcd for  $[Cp^{biph}Ir(bpy-OH)Cl]PF_6$  (834.21): C, 44.63; H, 3.50; N, 3.36. Found: C, 44.51; H, 3.46; N, 3.41. MS: m/z 689.00  $[Cp^{biph}Ir(bpy-OH)Cl]^+$ . IR (KBr pellets, cm<sup>-1</sup>): 3445 (-OH), 1036 (C-O).

[Cp<sup>biph</sup>Ir(bpy-OCH<sub>3</sub>)Cl]PF<sub>6</sub> (3). The synthesis was performed as for 1 using [Cp<sup>biph</sup>IrCl<sub>2</sub>]<sub>2</sub> (109 mg, 0.1 mmol) and 4, 4'-dimethoxy-2, 2'-bipyridine (52.5 mg, 0.24

mmol). Yield: 127 mg (72%). A single crystal was obtained by slow evaporation of a methanol/diethyl ether solution at room temperature. <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>):  $\delta$  8.48 (d, J = 6.6 Hz, Ar-*H*, 2H), 8.42 (d, J = 2.7 Hz, Ar-*H*, 2H), 7.82 (d, J = 8.3 Hz, Ar-*H*, 2H), 7.77 (d, J = 7.4 Hz, Ar-*H*, 2H), 7.61 (d, J = 8.2 Hz, Ar-*H*, 2H), 7.51 (t, J = 7.7 Hz, Ar-*H*, 2H), 7.44-7.39 (m, Ar-*H*, 3H), 4.07 (s, OC*H*<sub>3</sub>, 6H), 1.76 (s, Cp<sup>biph</sup>-*H*, 6H), 1.71 (s, Cp<sup>biph</sup>-*H*, 6H). Anal. Calcd for [Cp<sup>biph</sup>Ir(bpy-OCH<sub>3</sub>)Cl]PF<sub>6</sub> (862.27): C, 45.97; H, 3.86; N, 3.25. Found: C, 45.85; H, 3.81; N, 3.28. MS: m/z 717.02 [Cp<sup>biph</sup>Ir(bpy-OCH<sub>3</sub>)Cl]<sup>+</sup>. IR (KBr pellets, cm<sup>-1</sup>): 1384 (C-H), 1047 (C-O).

 $[Cp^{biph}Ir(bpy-Cl)Cl]PF_6$  (5). The synthesis was performed as for 1 using  $[Cp^{biph}IrCl_2]_2$  (109 mg, 0.1 mmol) and 4, 4'-dichloro-2, 2'-bipyridine (55.0 mg, 0.24 mmol). Yield: 144 mg (81%). A single crystal was obtained by slow evaporation of a methanol/diethyl ether solution at room temperature. <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>):  $\delta$  9.19 (d, J = 2.2 Hz, Ar-H, 2H), 8.66 (d, J = 6.2 Hz, Ar-H, 2H), 8.00 (dd, J = 6.2, 2.2 Hz, Ar-H, 2H), 7.82 (d, J = 8.4 Hz, Ar-H, 2H), 7.79-7.76 (m, Ar-H, 2H), 7.62 (d, J = 8.3 Hz, Ar-H, 2H), 7.51 (t, J = 7.6 Hz, Ar-H, 2H), 7.43 (t, J = 7.4 Hz, Ar-H, 1H), 1.78 (s, Cp<sup>biph</sup>-H, 6H), 1.72 (s, Cp<sup>biph</sup>-H, 6H). Anal. Calcd for  $[Cp^{biph}Ir(bpy-Cl)Cl]PF_6$  (871.09): C, 42.74; H, 3.12; N, 3.22. Found: C, 42.63; H, 3.09; N, 3.24. MS: m/z 724.90  $[Cp^{biph}Ir(bpy-Cl)Cl]^+$ . IR (KBr pellets, cm<sup>-1</sup>): 558 (C-Cl).

 $[Cp^{biph}Ir(bpy-NO_2)CI]PF_6$  (6). The synthesis was performed as for 1 using  $[Cp^{biph}IrCl_2]_2$  (108 mg, 0.1 mmol) and 4, 4'-dinitro-2, 2'-bipyridine (60.2 mg, 0.24 mmol). Yield: 62 mg (35%). A single crystal was obtained by slow evaporation of a methanol/diethyl ether solution at room temperature. <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>):  $\delta$  8.48 (d, J = 6.5 Hz, Ar-H, 2H), 8.42 (d, J = 2.5 Hz, Ar-H, 1H), 7.82 (d, J = 8.1 Hz, Ar-H, 2H), 7.77 (d, J = 7.6 Hz, Ar-H, 2H), 7.60 (d, J = 8.1 Hz, Ar-H, 2H), 7.51 (t, J = 7.5 Hz, Ar-H, 3H), 7.45-7.37 (m, Ar-H, 3H), 1.80–1.75 (m, Cp<sup>biph</sup>-H, 6H), 1.74-1.69 (m, Cp<sup>biph</sup>-H, 6H). Anal. Calcd for  $[Cp^{biph}Ir(bpy-NO_2)CI]PF_6$  (892.21): C, 41.73; H, 3.05; N, 6.28. Found: C, 41.64; H, 3.02; N, 6.31. MS: m/z 747.10  $[Cp^{biph}Ir(bpy-NO_2)CI]^+$ . IR (KBr pellets, cm<sup>-1</sup>): 1619 (NO<sub>2</sub>), 1384 (NO<sub>2</sub>), 1315 (C-N), 1256 (N-O).

#### ASSOCIATED CONTENT

#### **Supplementary Materials**

Full experimental details for the synthesis of ligands, NMR, IR and MS spectra of all complexes, crystallographic data for 1, 3, and 5, the data for nucleobase studies, aqueous chemistry, cancer cell studies, DNA reaction and NADH reaction kinetics. CIF and checkCIF files for 1, 3 and 5. CCDC numbers of 1, 3 and 5 are 1569686,

1569665, and 1569685, respectively. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data\_request/cif.

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# Notes

The authors declare no competing financial interests.

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