Cyclometalated iridium(III) complexes as mitochondria-targeted anticancer agents

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Abstract

Four cyclometalated iridium(III) complexes $[\text{Ir}(\text{dfppy})_2(\text{L})]^+$ (dfppy = 2-(2,4-difluorophenyl)pyridine, L = 6-(pyridin-2-yl)-1,3,5-triazine-2,4-diamine, **Ir1**; 6-(isoquinolin-1-yl)-1,3,5-triazine-2,4-diamine, **Ir2**; 6-(quinolin-2-yl)-1,3,5-triazine-2,4-diamine, **Ir3**; 6-(isoquinolin-3-yl)-1,3,5-triazine-2,4-diamine, **Ir4**) have been synthesized and characterized. Distinct from cisplatin, **Ir1–Ir4** could specifically target mitochondria and induced apoptosis against various cancer cell lines, especially for cisplatin resistant cells. ICP-MS results indicated that **Ir1–Ir4** were taken up *via* different mechanism for cancer cells and normal cells, which resulted in their high selectivity. The structure-activity relationship and signaling pathways were also discussed.

Keywords: Cyclometalated iridium(III) complexes; Mitochondria-targeted; Anticancer; Apoptosis;

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1. Introduction

Since the clinical success of cisplatin, carboplatin and oxaliplatin, the therapeutic value of metal-based drugs has long been established [1]. DNA is generally accepted to be the main target of platinum-based anticancer drugs, however, these platinum compounds suffer from two main disadvantages: they are inefficient against platinum-resistant tumors, and they have severe side effects such as neurotoxicity, kidney problems and allergic reactions. These drawbacks have also stimulated the development of non-platinum metal-based therapeutics [2-4].

Organometallic iridium complexes have recently been found to be alternatives to platinum-based anticancer agents because of their rich synthetic chemistry, variable oxidation states that are accessible under physiological conditions and kinetically stable [5-6]. Moreover, their multiple mechanisms of anticancer action may be distinct from those of platinum-based drugs. Meggers and co-workers investigated the binding of iridium to pyridocarbazole, which are known to be a ATP competitive inhibitor that targets the ATP-binding site of protein kinases [7]. Serum albumin is another target of anticancer iridium compounds that reported by Lo and co-workers [8]. Indole derivative, which is known to bind to BSA, was introduced into iridium complexes and were found to be highly cytotoxic toward HeLa cells. In addition to protein, various organometallic iridium have been found to bind DNA through intercalation or to form DNA-Ir adducts [9]. Interestingly, most organometallic ligands of the reported iridium anticancer complexes are metallocenes [10-12], half-sandwich [13-15], carbene- [16], CO- [17], or π -ligands [18]. Traditional cyclometalated polypyridyl-like ligands (CNN) have attracted

much less attention.

As one of the most important cell signaling center, mitochondria are essential organelles required for cellular energy production and are involved in many other cellular activities, one of which is to produce reactive oxygen species (ROS) and leads to mitochondria-mediated apoptosis [19-21]. Our previous works showed that mitochondria is the main target of cyclometalated iridium(III) complexes [22-27]. Herein, four novel cyclometalated iridium(III) complexes were developed as mitochondria-targeted theranostic agents. The mitochondria-specific target, cellular uptake and antitumor activity, as well as cell-cycle arrest and apoptosis signaling pathways were studied. The unique structure and characteristic of Ir(III) complex designed in this investigation may contribute to the future development of improved chemotherapeutics.

2. Experimental

2.1. Materials and measurements

All reagents were purchased from commercial sources and used without further purification unless otherwise specified. Chromatographic separations were performed on neutral aluminum oxide. All solvents were of analytical grade. All buffer components were of biological grade and used as received. $\text{IrCl}_3 \cdot 3\text{H}_2\text{O}$, 2-(2,4-difluorophenyl)pyridine (dfppy), 2-ethoxyethanol, 2-cyano-pyridine, isoquinoline-1-carbonitrile, 2-cyanoquinoline, isoquinoline-3-carbonitrile and dicyandiamide were purchased from Sigma Aldrich and were used without further purification. Four ligands, 6-pyridyl-1,3,5-triazine-2,4-diamine (PyTNH),

6-(isoquinolin-1-yl)-1,3,5-triazine-2,4-diamine (1-IQTNH), 6-(quinolin-2-yl)-1,3,5-triazine-2,4-diamine (2-QTNH) and 6-(isoquinolin-3-yl)-1,3,5-triazine-2,4-diamine (3-IQTNH) were prepared according to our previously reported methods [28-29]. All the complexes were dissolved in DMSO immediately preceding the experiments; the calculated quantities of the drug solutions were then added to the appropriate medium to yield a final DMSO concentration of less than 1% (v/v).

Microanalysis (C, H, and N) was carried out using a Perkin-Elmer 240Q elemental analyzer. Electrospray mass spectra (ESI-MS) were recorded on a LCQ system (Finnigan MAT, USA). The expected and measured isotope distributions were compared. The ^1H NMR spectra were recorded on a Bruker AVANCE 400 spectrometer (400 MHz). All chemical shifts are reported relative to tetramethylsilane (TMS).

2.2. Synthesis of the iridium(III) complexes **Ir1–Ir4**

The complexes **Ir1–Ir4** were synthesized in a similar manner to previously reported methods [22-23]. Briefly, a mixture of 2-ethoxyethanol and water (3:1, v/v) was added to a flask containing $\text{IrCl}_3 \cdot 3\text{H}_2\text{O}$ (1.0 mmol) and the cyclometalated ligand dfppy (2.5 mmol). The mixture was refluxed for 24 h. After cooling, the yellow precipitate was filtered to give crude cyclometalated Ir(III) chloro-bridged dimer [30]. The chloro-bridged dimer (0.20 mmol) and four ligands (PyTNH, 1-IQTNH, 2-QTNH and 3-IQTNH, 0.4 mmol respectively) were placed in a 100 mL round bottomed flask with 48 mL of methanol and dichloromethane (3:1, v/v). The mixture was heated at 65°C for 8 h under argon. The solution was filtered, and the precipitate was washed three times (2 mL) with methanol.

The filtrate and washings were combined and reduced by evaporation to a volume of 1 mL. The product was purified by column chromatography on alumina using acetonitrile as the eluant (yield: 62 – 74%). Then, the iridium complexes were further characterized by elemental analysis, ^1H NMR and ESI-MS measurements. The structural parameters of **Ir4** are described in detail in the Supporting Information.

Ir1. Yield: 73%. ^1H NMR (400 MHz, DMSO- d_6) δ 8.58 (d, J = 7.7 Hz, 1H), 8.47 (dd, J = 5.8, 0.8 Hz, 1H), 8.36 (dd, J = 4.3, 2.4 Hz, 1H), 8.35 – 8.32 (m, 1H), 8.30 (d, J = 8.5 Hz, 1H), 8.15 – 8.06 (m, 2H), 8.01 (s, 1H), 7.94 (s, 1H), 7.91 (s, 1H), 7.80 (m, 1H), 7.79 – 7.75 (m, 1H), 7.62 (dd, J = 5.8, 0.8 Hz, 1H), 7.40 (m, 1H), 7.35 (m, 1H), 7.00 (m, 2H), 5.64 (dd, J = 8.2, 2.3 Hz, 1H), 5.46 (dd, J = 8.4, 2.3 Hz, 1H), 4.97 (s, 1H). ESI-MS: m/z = 760.80 ($[\text{M-Cl}]^+$). Elemental Analysis: Anal. Calcd. for $\text{C}_{30}\text{H}_{20}\text{ClF}_4\text{IrN}_7(\%)$: C, 45.26, H, 2.53, N, 14.07; found(%): C, 45.17, H, 2.44, N, 14.15.

Ir2. Yield: 67%. ^1H NMR (400 MHz, DMSO- d_6) δ 10.51 (d, J = 8.8 Hz, 1H), 8.54 (dd, J = 5.8, 0.8 Hz, 1H), 8.37 (d, J = 8.5 Hz, 1H), 8.29 (d, J = 8.2 Hz, 2H), 8.18 (d, J = 6.1 Hz, 1H), 8.17 – 8.09 (m, 2H), 8.05 (d, J = 8.9 Hz, 1H), 8.02 (d, J = 1.2 Hz, 1H), 8.00 (d, J = 1.2 Hz, 1H), 7.99 – 7.95 (m, 1H), 7.95 – 7.90 (m, 1H), 7.81 (m, 1H), 7.73 (t, J = 5.9 Hz, 1H), 7.34 (m, 1H), 7.29 (m, 1H), 7.05 – 6.95 (m, 2H), 5.62 (m, 1H), 5.50 (m, 1H), 5.16 (s, 1H). ESI-MS: m/z = 810.90 ($[\text{M-Cl}]^+$). Elemental Analysis: Anal. Calcd. for $\text{C}_{34}\text{H}_{22}\text{ClF}_4\text{IrN}_8(\%)$: C, 50.37, H, 2.74, N, 13.82; found(%): C, 50.29, H, 2.64, N, 13.77.

Ir3. Yield: 62%. ^1H NMR (400 MHz, DMSO- d_6) δ 8.93 (d, J = 8.7 Hz, 1H), 8.70 (d, J = 8.6 Hz, 1H), 8.48 (d, J = 5.7 Hz, 1H), 8.43 (d, J = 8.5 Hz, 1H), 8.15 (d, J = 8.5 Hz, 2H), 8.12 (dd, J = 8.9, 4.6 Hz, 1H), 8.08 (d, J = 5.9 Hz, 2H), 7.98 (s, 1H), 7.97 – 7.93 (m, 1H),

7.88 (s, 1H), 7.72 (d, $J = 9.0$ Hz, 1H), 7.67 (dd, $J = 11.5, 4.3$ Hz, 1H), 7.38 (m, 1H), 7.34 – 7.31 (m, 1H), 7.31 – 7.28 (m, 1H), 7.12 – 7.04 (m, 1H), 7.01 – 6.93 (m, 1H), 5.65 (dd, $J = 8.5, 2.3$ Hz, 1H), 5.61 (dd, $J = 8.3, 2.3$ Hz, 1H), 4.92 (s, 1H). ESI-MS: $m/z = 810.85$ ($[M-Cl]^+$). Anal. Calcd. for $C_{34}H_{22}ClF_4IrN_8(\%)$: C, 50.37, H, 2.74, N, 13.82; found(%): C, 50.41, H, 2.80, N, 13.75.

Ir4. Yield: 74%. 1H NMR (400 MHz, DMSO- d_6) δ 9.14 (s, 1H), 8.51 (dd, $J = 5.8, 0.8$ Hz, 1H), 8.46 (s, 1H), 8.37 (dd, $J = 8.2, 5.1$ Hz, 2H), 8.30 (d, $J = 8.5$ Hz, 1H), 8.20 (d, $J = 8.0$ Hz, 1H), 8.11 (t, $J = 7.7$ Hz, 1H), 8.08 – 8.05 (m, 1H), 8.05 – 8.02 (m, 1H), 7.95 – 7.84 (m, 4H), 7.77 – 7.72 (m, 1H), 7.37 (m, 1H), 7.26 (m, 1H), 7.07 – 6.97 (m, 2H), 5.69 – 5.60 (m, 1H), 5.52 (dd, $J = 8.3, 2.3$ Hz, 1H), 5.04 (s, 1H). ESI-MS: $m/z = 810.80$ ($[M-Cl]^+$). Anal. Calcd. for $C_{34}H_{22}ClF_4IrN_8(\%)$: C, 50.37, H, 2.74, N, 13.82; found(%): C, 50.33, H, 2.69, N, 13.79.

2.3. X-Ray crystallography

Single crystals of the complex **Ir3** suitable for an X-ray crystallographic study were grown from acetonitrile–toluene (1 : 1, v/v) at room temperature. A yellow crystal with the dimensions $0.12 \times 0.1 \times 0.09$ mm³ was recorded on a Rigaku R-Axis SPIDER IP diffractometer (MoK α , $\lambda = 0.71073$ Å) at 293(2) K. An absorption correction was applied with the SADABS program [31]. The structure solution and full-matrix least-squares refinement based on F² for **Ir3** were performed with the SHELXS 97 and SHELXL 9734 program packages, respectively [32]. All the non-hydrogen atoms were refined anisotropically. Hydrogen atoms of the organic ligands were generated geometrically

(C–H 0.96 Å). Detailed crystallographic data for the crystal structural analysis have been deposited with the Cambridge Crystallographic Data Centre (1448028) contains the supplementary crystallographic data for the present paper.

2.4. Cell Culture Conditions and in vitro Cytotoxicity Assay

MCF-7, NCI-H460, HepG2, Bel-7402, L-02, HeLa and HCT-116 cells were maintained as monolayer cultures in DMEM. The A549 and A549R cells were maintained in RPMI 1640. Exponentially growing cells were seeded in triplicate into 96-well plates at 1×10^4 cells/well. After incubation for 24 h, the cells were treated with increasing concentrations of the tested complexes for 48 h. The *in vitro* cytotoxicity assay was performed by using the MTS kit following the manufacturer's protocol (Promega, WI, US). The absorbance of the samples was measured at 490 nm in an ELISA reader (BioTek Instruments, Winooski, VT).

2.5. Cellular uptake and the degree of localization

For uptake studies, exponentially growing A549R and LO2 cells were harvested, and the resulting single-cell suspension was plated in 100 mm tissue culture plates (Costar) at 1×10^6 cells/plate. After 24 h at 37 °C, the cells were incubated with 2.5 µM Ir(III) complexes for 6 h at 37 °C. The cells were rinsed with PBS, detached with trypsin, counted and divided into two portions. In the first portion, the cells were keep integrity; in the second portion, the mitochondrial was extracted using a mitochondrial extraction kit (Pierce, Thermo). All samples were digested in 60% HNO₃ at room temperature for 24 h.

Each sample was then diluted with doubly-distilled water to obtain 2% HNO₃ sample solutions [33]. The iridium concentrations in the two portions were determined by ICP-MS (Thermo Elemental Co., Ltd.). Data are reported as the mean $\pm$ standard deviation (n = 3).

2.6. Temperature and inhibitor studies [34]

A549R cells were detached from the culture and were preincubated with 50 mM 2-deoxy-D-glucose and 5 μ M oligomycin in serum-free DMEM for 1 h before incubation of 2.5 μ M **Ir1–Ir4** with inhibitor in the fresh media for 6 h at 4 °C or 37 °C. The cells were washed with PBS solution and then trypsinized and processed for ICP-MS analysis.

2.7. Cell cycle and Annexin-V analysis

After treated with **Ir1–Ir4** (2.5 μ M) for 24 h, A549R cells were harvested and centrifuged (10 min at 800 g) and fixed in 2 mL of 70% aqueous ethanol (v/v). After an incubation period of at least 12 h at -20 °C, cells were centrifuged (10 min at 800 g) and washed twice with ice-cold PBS. The cells were re-suspended in 200 μ L staining solution containing PI (10 μ g/mL) and DNase-free RNase (100 μ g/mL) and analyzed by a BD FACSCaliburTM cytometer (Becton Dickinson, Heidelberg, Germany). The number of cells analyzed for each sample was 10000, and the experiments were repeated at least three times under identical conditions. Data were collected by BD CellQuestTM Pro software and analyzed by ModFit LT 2.0 software.

Annexin V and propidium iodide (PI) co-staining of the apoptotic membranes was

performed by using the annexin V-FITC apoptosis detection kit following the manufacturer's protocol (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.). A549R cells seeded into 35 mm glass-bottom dishes (Corning) were treated with **Ir1–Ir4** (2.5 μ M) for 24 h. After washed twice with PBS, the cells were stained with annexin V-FITC for 10 min. Cells were washed twice with PBS and then were stained with PI and measured by a BD FACSCaliburTM cytometer with an excitation wavelength of 488 nm and an emission wavelength of 530 ± 20 nm and 610 ± 20 nm. Annexin-V positive cells were considered apoptotic.

2.8. Analysis of mitochondrial membrane potential

Mitochondrial dysfunction was determined by measuring changes in the mitochondrial membrane potential (MMP, $\Delta\Psi_m$) using an inverted fluorescence microscope and flow cytometry after staining live cells with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) [35]. A549R cells were treated with 2.5 μ M **Ir1–Ir4** for 24 h. For flow cytometry analysis, A549R cells were re-suspended at 1×10^6 /mL in pre-warmed JC-1 working buffer containing 5 μ g/mL JC-1 and incubated for 15 min in a 37 °C, 5% CO₂ incubator. Subsequently, the cells were washed in assay buffer and directly analyzed in a BD FACSCaliburTM cytometer (Becton Dickinson, Heidelberg, Germany). Photomultiplier settings were adjusted to detect JC-1 monomer fluorescence signals on the FL1 detector (green fluorescence, centered around 525 nm) and JC-1 aggregate fluorescence signals on the FL2 detector (red fluorescence, centered around 590 nm). The relative monomer (green) fluorescence intensity values

were used for data presentation.

2.9. Caspase -3/7 and caspase 9 activities assay

A549R cells were treated with **Ir1–Ir4** (2.5 μ M) for 24 h, and the caspase -3/7 and caspase 9 activities were determined using the Caspase-Glo assay (Promega, WI, US) according to the manufacturer's instructions [4].

2.10. Intracellular reactive oxygen species (ROS) measurement

After treatment with 2.5 μ M **Ir1–Ir4** for 24 h, A549R cells were incubated with 10 μ M DCFH-DA (Sigma-Aldrich) for 20 min at 37 °C [36]. The fluorescence intensity of cells was measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ), confocal microscope (Leica Microsystems, Wetzlar, Germany), and microplate analyzer (Infinite M200, TECAN, Switzerland) with excitation set at 488 nm and emission at 530 ± 20 nm. For ROS inhibitors assay, Tiron (5 mM) and NAC (10 mM) were applied 1 h before **Ir1–Ir4** (2.5 μ M) treatment and kept in the medium during **Ir1–Ir4** treatment until the cells were analyzed.

3. Results and discussion

3.1. Synthesis and characterization

It is known that diamino-1,3,5-triazines are a class of compounds possessing diverse bioactivities such as anticancer, anti-inflammation, anti-allergy and offering a protective effect against gastric lesions [37-41]. 2,4-diamino-1,3,5-triazine derivatives were easily

prepared following the method established by Case [42]. The preparations of the **Ir1–Ir4** (Fig. 1) complexes were carried out through bridge splitting reactions of the dinuclear precursors $[\text{Ir}(\text{dfppy})_2\text{Cl}]_2$ with the 2,4-diamino-1,3,5-triazine ligands in a stoichiometric amount [22]. The synthesized complexes were characterized with ESI-MS, elemental analysis and ^1H NMR spectroscopy (Fig. S1–S4 in Supplementary Information).

The molecular structures of **Ir3** was also confirmed by single-crystal X-ray diffraction analysis (Fig. 2). The crystal data and structural refinements are shown in Table S1. Selected bond lengths and angles are presented in Table S2. The coordination of the iridium atom of **Ir3** is distorted octahedral, with the largest deviation observed in the bite angle (75.23°) of the 2,4-diamino-1,3,5-triazine derivative ligand (Table S2). The cyclometalated C atoms of the dfppy ligands are in a mutually *cis* arrangement, and their high trans influence leads to Ir–N (2-QTNH) distances ($2.194(2)$ Å), which are longer than the corresponding distances ($2.006(5)$ Å) in the cyclometalated ligand [43].

3.2. *In vitro* cytotoxicity

To explore the antitumor potential of **Ir1–Ir4**, a panel of cell lines representing a range of tumor types, including MCF-7, NCI-H460, HepG2, Bel-7402, L-02, HeLa, HCT-116, A549 and A549R (cisplatin-resistant A549 cells) was treated with various concentrations of **Ir1–Ir4** or cisplatin (positive control) for 48 h. As human normal cells, LO2 was set as the control group. *In vitro* cytotoxicity experiment were performed by using the MTS kit following the manufacturer's protocol. The resulting IC_{50} values for the tested compounds are shown in Table 1. **Ir1** exhibited a broad spectrum of inhibition on various human

cancer cells, with IC_{50} values ranging from 8.68 to 19.0 μM , which were comparable with those of cisplatin ($IC_{50} = 14.7 \sim 87.2 \mu M$), indicating cytotoxic effects on cancer cells. As isomers, **Ir2** and **Ir3** possessed similar IC_{50} values and were more potent than **Ir1** against all cancer cells screened. Interestingly, unexpected excellent anticancer properties for **Ir4** were observed. HeLa cells was especially susceptible to **Ir4**, with a lower IC_{50} (0.86 μM) than that of **Ir2** (6.44 μM) and **Ir3** (5.96 μM), although **Ir4** is the isomer of **Ir2** and **Ir3** as well. In addition, all iridium complexes were sensitized to cisplatin-resistance cancer cells A549R, implying the existence of different anticancer mechanism. Therefore, A549R cells were selected for further researches.

Possessing selectivity between human cancer and normal cells is important in developing cancer chemotherapy. As shown in Table 1, the IC_{50} values of **Ir1–Ir3** in LO2 cells were 47.2 ± 3.2 , 40.2 ± 4.1 and $38.6 \pm 2.8 \mu M$, higher than those of cancer cells and similar with that of cisplatin ($46.4 \pm 4.8 \mu M$). Analogous phenomenon was observed for **Ir4**. It should be pointed out that, despite **Ir4** exhibited much higher cytotoxicity in cancer cell lines than **Ir1–Ir3**, their IC_{50} value for normal cells were similar.

3.3. Cellular uptake and the degree of localization

As reported, the cytotoxicity of transition metal-based anticancer drugs are dramatically influenced by their cellular uptake [44]. Therefore, the difference of *in vitro* cytotoxic activity for **Ir1–Ir4**, as well as the selectivity between cancer cells and normal cells, indicated that the cellular uptake of **Ir1–Ir4** may be different. The uptake of **Ir1–Ir4** by the A549R cells and LO2 cells were quantified by ICP-MS. Cells were

incubated with 2.5 μM of **Ir1–Ir4** for 6 h, and excess dye was washed away by the buffer solution. Four iridium(III) complexes could cross the cellular membranes to eventually reach the inside of the cell. ICP-MS measurements of the **Ir1–Ir4** content gave 7.63 ± 0.42 , 8.23 ± 0.60 , 8.06 ± 0.38 and 7.87 ± 0.54 fg/cell for A549R cells, while those were 2.38 ± 0.30 , 2.96 ± 0.24 , 2.82 ± 0.27 and 3.02 ± 0.15 fg/cell for LO2 cells (Fig. 3), indicated that the cellular uptake mechanism may be different.

The key factors involved in iridium(III) complex **Ir1–Ir4** internalization were investigated in detail to further confirm our speculate. Generally speaking, small molecules can be uptake through energy-dependent (endocytosis, active transport) and energy-independent (passive diffusion) pathways. As shown in Fig. 4, low temperature (4 $^{\circ}\text{C}$) incubation led to inhibition of cellular uptake by 37%, 40%, 36% and 33% for A549R cells, but shown little influence on LO2 cells. This interesting result indicated that energy-independent pathway, such as passive diffusion, is the cellular uptake mechanism for LO2 cells but is not involved in that of A549R cells. This conclusion was further support by metabolic inhibition results. Cells were pretreated with two metabolic inhibitors, oligomycin and 2-deoxy-D-glucose, which deplete the cell's energy and diminish energy-dependent uptake, and then were incubated with 2.5 μM of **Ir1–Ir4**. As determined by ICP-MS (Fig. 4), when active transport was inhibited, the intracellular iridium amount for A549R cells was significantly suppressed but had no effect on LO2 cells.

The distribution of localization characteristics is another key point that influences the cytotoxicity effect [45-46]. Out of expect, no significant difference was observed in the

specificity to mitochondria between these four iridium complexes. For A549R cells, the ratio $I_{\text{mito/cell}}$ between the mitochondria and whole cell was 85.3%, 86.2%, 82.7% and 88.1%. Similar results are observed for LO2 cells and the $I_{\text{mito/cell}}$ was 87.7%, 84.6%, 85.4% and 85.3%, respectively. This result is not consistent with the MTS experiment that **Ir4** possessed the highest *in vitro* cytotoxicity. Thus, we speculated that there were some unknown interactions between **Ir4** and mitochondria.

3.4. Apoptosis and cell cycle arrest

It is known that the externalization of the phosphatidylserine membrane is the characteristics of cells undergoing apoptosis [47]. Annexin-V/PI staining experiment was performed. In contrast to vehicle-treated cells, after treatment with **Ir1–Ir4** (2.5 μM) for 24 h, the percentage of A549R cells stained positive for annexin-V was $11.8 \pm 0.6\%$, $12.2 \pm 1.1\%$, $14.7 \pm 0.8\%$ and $21.7 \pm 1.8\%$, respectively (Fig. 5a). This result consists with the *in vitro* cytotoxicity.

Because cell cycle arrest phenomenon has been observed in various metal-based anticancer complexes induced cell apoptosis, therefore, the effect of **Ir1–Ir4** on the cell cycle was also investigated. Fig. 5b shown that exposure of the A549R cells to 2.5 μM **Ir1–Ir4** for 24 h resulted in marked increase in the proportion of apoptotic cells as reflected by the sub-diploid peak. The values of sub-G1 varied from 6.7% to as high as 27.5%, which consisted with the *in vitro* cytotoxicity. However, it is interesting that the cell cycle arrest induced by **Ir1–Ir4** were not significant. The proportion of cell cycle varied irregularly. For example, the value of G0/G1 was 54.2% for the control cells, and

this proportion reduced to 48.5% and 41.9% for **Ir1–Ir2**, but increased to 58.7% for **Ir3** and 62.3% for **Ir4**. In addition, the percent of G2 period was 17.8%, 22.6%, 25.0%, 21.1% and 9.8% for control and **Ir1–Ir4** group, respectively. These results indicate that the anti-proliferative activities of the **Ir1–Ir4** were mainly caused by apoptosis rather than cell cycle arrest [48-50].

3.5. The induction of mitochondrial dysfunction

Due to the fact that mitochondrial dysfunction is involved in cell apoptotic death. Mitochondrial dysfunction induced by **Ir1–Ir4** was determined by measuring changes in the mitochondrial membrane potential (MMP, $\Delta\Psi_m$). JC-1 is cationic dyes that exhibit potential-dependent accumulation in mitochondria. Mitochondrial depolarization is indicated by a decrease in the red (~590 nm)/green (~525 nm) fluorescence intensity ratio. The representative JC-1 red/green signal ratios recorded by flow cytometry (Fig. 6). After 24 h of incubation, treatment with 2.5 μ M **Ir1–Ir4** decreased the JC-1 red/green signal ratio from 31.2 ± 2.0 (control) to 12.6 ± 0.8 , 1.58 ± 0.2 , 1.41 ± 0.1 and 0.32 ± 0.1 , respectively.

The loss of MMP was also evidenced by the activities of caspase-9 and caspase-3/7. When mitochondrial membrane permeability transition, cytochrome *c* may be released from mitochondria and induced the activating of caspase 9, then subsequently initiates a caspase cascade involving caspase-3. caspase-3 is known to be the biochemical hallmark of both early and late-stage apoptosis. Treated with 2.5 μ M **Ir1–Ir4** for 24 h, the activities of caspase-9 and caspase-3/7 were detected using the Caspase-Glo assay kit (Promega,

US). As shown in Fig. 7a, significant increases in the activities of caspase-9 and caspase-3/7 were observed. Possessing the best *in vitro* anti-proliferative activity, the treatment of **Ir4** resulted in a 5.4-fold and 6.2-fold increase in the fluorescence signal for caspase-9 and caspase-3/7 when compared with control.

3.6. Intracellular reactive oxygen species (ROS) accumulation

Induced ROS accumulation is one of the important characters that involved in various metal-based anticancer complexes. ROS accumulation was quantified by the DCFH-DA (2',7'-dichlorofluorescein-diacetate) staining. DCFH-DA can be oxidized by the presence of endogenously generated ROS and release the fluorophore 2',7'-dichlorofluorescein (DCF). Treatment with 2.5 μ M **Ir1–Ir4** for 24 h resulted in a 4.4, 5.1, 5.6 and 7.8 fold increase in the fluorescence signal compared with control (Fig. 7a). To further examine the role of ROS generation in **Ir1–Ir4** induced apoptotic cell death, the effects of two ROS scavengers, tiron (4,5-dihydroxy-1,3- benzenedisulfonic acid disodium salt) and NAC (N-acetylcysteine), on **Ir1–Ir4** induced apoptosis were investigated. Tiron and NAC was nontoxic at the tested concentrations for A549R. The presence of either tiron or NAC significantly suppressed the **Ir1–Ir4** induced apoptosis as demonstrated by the drastic decrease in Annexin-V positive labeling (Fig. 7b).

4. Conclusions

Four cyclometalated iridium(III) complexes with 2,4-diamino-1,3,5-triazine derivatives (**Ir1–Ir4**) were successfully developed as novel anticancer agent. *In vitro* cytotoxicity

experiments demonstrated that in addition to possessing higher cytotoxic potency than the widely used clinical chemotherapeutic agent cisplatin, **Ir1–Ir4** exhibited high selectivity between tumor cells and normal cells. This essential selectivity for developing anticancer drugs is resulted from the different mechanisms of entering tumor cells and normal cells. As evidenced by ICP-MS, energy-independent pathway is the cellular uptake mechanism for LO2 cells while **Ir1–Ir4** were taken up by A549R cells *via* energy-dependent pathway. Moreover, the intracellular iridium amounts as well as the distribution of localization of **Ir1–Ir4** in A549R were similar. Interestingly, **Ir4** shows much more potential than **Ir2** and **Ir3**, even though they are isomers. Therefore, some unknown interactions between **Ir4** and mitochondria are suggested and further study is required. Signaling pathways analysis demonstrated that these complexes induced apoptosis *via* the mitochondrial pathway. Treatment of A549R cells with **Ir1–Ir4** could result in generation of ROS, prominent depletion of $\Delta\Psi_m$ and activation of Caspase 9 and Caspase 3/7. We hope that these results will prove of value in further understanding the cellular uptake of iridium complexes and may contribute to the future development of improved chemo-therapeutics against human cancers.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at .

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Figure captions:

- Fig. 1** Chemical structures of complexes **Ir1–Ir4**.
- Fig. 2** ORTEP diagrams of the Ir(III) complexes **Ir3**. For clarity, the solvent molecules, the hydrogen atoms, and the counter anions are omitted.
- Fig. 3** ICP-MS quantification of the internalized iridium by the A549R and LO2 cells. Cells were treated with 2.5 μ M of **Ir1–Ir4** at 37 °C for 6 h.
- Fig. 4** The cellular uptake mechanism study. A549R cells (**a**) and LO2 cells (**b**) were pretreated with 50 mM 2-deoxy-D-glucose and 5 μ M oligomycin in serum-free DMEM for 1 h before incubation of 2.5 μ M **Ir1–Ir4** with inhibitor in the fresh media for 6 h at 4 °C or 37 °C, then collected and analyzed using ICP-MS.
- Fig. 5** (**a**) Histograms of A549R cells stained with annexin V-FITC conjugate after treatment with 2.5 μ M **Ir1–Ir4** for 24 h. FITC fluorescence was analysed in the FL1 channel with excitation at 488 nm and emission at 530 ± 15 nm. (**b**) A549R cells were treated with **Ir1–Ir4** (2.5 μ M) for 24 h, then collected and cell cycle was analyzed using flow cytometry. Apoptotic cells were calculated by measuring the area of the sub-G1 peak.
- Fig. 6** The Effects of **Ir1–Ir4** on the mitochondrial membrane potential. Cells were treated with 2.5 μ M **Ir1–Ir4** for 24 h, stained with JC-1 dye, and analysed by flow cytometry. Red fluorescence: hyperpolarized mitochondria; green fluorescence: depolarized mitochondria.
- Fig. 7** (**a**) A549R cells were treated with 2.5 μ M **Ir1–Ir4** for 24 h. Caspase-9, caspase-3/7 activity and intracellular ROS expressed as the relative fluorescence intensities measured using a microplate reader; (**b**) Attenuating the ROS levels by tiron (5 mM) and NAC (10 mM) reduced the apoptosis population upon treatment with 2.5 μ M **Ir1–Ir4**

for 24 h.

Table 1. IC₅₀ values for the tested compounds toward various cell lines.^a

Origin	Cell Line	IC ₅₀ (μM)				
		Ir1	Ir2	Ir3	Ir4	cisplatin
Breast	MCF-7	14. 6 ± 1.0	12.1 ± 1.2	12.6 ± 0.9	4.61 ± 0.5	22.8 ± 4.2
Lung	A549	17.5 ± 1.2	10.1 ± 0.7	9.53 ± 0.5	2.77 ± 0.2	16.5 ± 2.5
	A549R	16.2 ± 2.2	11.5 ± 0.8	10.0 ± 0.6	2.38 ± 0.4	87.2 ± 4.3
	NCI-H460	16.4 ± 1.7	13.4 ± 1.2	15.4 ± 0.8	5.64 ± 0.3	22.1 ± 1.7
Liver	HepG2	19.0 ± 1.3	8.79 ± 0.5	9.41 ± 0.7	1.93 ± 0.3	18.7 ± 2.1
	Bel-7402	10. 8 ± 0.8	8.74 ± 0.7	9.02 ± 1.0	1.13 ± 0.1	27.7 ± 2.4
	L-02	47.2 ± 3.2	40.2 ± 4.1	38.6 ± 2.8	33.2 ± 4.1	46.4 ± 4.8
Cervix	HeLa	8.68 ± 0.4	6.44 ± 0.3	5.96 ± 0.3	0.86 ± 0.1	20.2 ± 3.6
Colorectal	HCT-116	13.2 ± 1.6	8.37 ± 0.6	9.11 ± 1.2	2.46 ± 0.3	14.7 ± 2.3

Figure 1

Figure 2

Figure 3

Figure 4

A549R

Figure 5

(a)

(b)

Figure 6

Control

Ir1

Ir2

Ir3

Ir4

FITC-A

PE-A

Figure 7