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A phosphorescent iridium(III) solvent complex for multiplex assays of cell death

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ABSTRACT

Cell death involves loss of transport function and physical integrity of the plasma membrane, and plays a critical role in many human diseases. At present, the development of an effective visualization tool to monitor cell death remains a significant challenge. Here, a cyclometalated iridium(III) solvent complex $[Ir(pdz)_2(H_2O)_2]^+[OTf]^-$ (**IrC1**) was designed and synthesized as a phosphorescent indicator of cell death. **IrC1** specifically stained the nuclei of dead cells over living cells rapidly (<10 min) and at low concentrations (10 μM), as observed using confocal luminescence microscopy. Moreover, the **IrC1** uptake behavior leads to its further application in quantifying the population of early apoptotic cells using flow cytometry. In particular, successful application in time-gated fluorescence microscopy by virtue of its microsecond lifetime rendered **IrC1** attractive as a luminescent probe. **IrC1** additionally exhibited excellent long-term photostability, in contrast to traditional dyes. We conclude that in combination with luminescent microscopy and flow cytometry, **IrC1** provides an effective, straightforward alternative to cell death assays.

1. Introduction

Determination of cell death is important in tissue culture and cytotoxicity studies [1–3]. The cell death assay is particularly employed in chemotherapy to select anti-cancer drugs [4,5]. The ability to visualize, track and quantitatively analyze cell death is essential for understanding the response to anti-cancer drugs. Fluorescence imaging is a powerful technique for the cell death assay, and many fluorescent indicator dyes are currently available. DNA intercalating dyes (ethidium monoazaide (EMA) [6–8], propidium iodide [9] and Annexin V [10,11]) are commonly used to exclude dead cells. PCR with ethidium monoazide is currently the accepted method for quantification of viable and dead cells in solution [12]. However, traditional fluorescent stains are limited to organic dyes with short fluorescence lifetime, which are disrupted by the presence of autofluorescence from biosamples and serious photobleaching.

Compared with pure organic fluorophores, metal-based emissive probes exhibit advantageous photophysical properties (such as relatively long lifetime, significant Stokes shift for easy separation of excitation and emission, and high photostability) [13-16]. Cyclometalated iridium (III) complexes are of particular interest due to the exclusive strong spin-orbit coupling of the iridium ion (coupling constant, $\xi_{Ir} = 3909\ cm^{-1}$), high luminescence quantum yield, and facile-tunable excitation and emission maxima from blue to near-infrared regions [17,18]. Currently, iridium(III) complexes are used as bioimaging reagents by several researchers, including our group [19–23]. However, iridium(III) complex application for the cell death assay has not been reported to date.

The development of new techniques and tools to reliably identify and quantitate cell death is crucial. Our group has focused on developing a visualization strategy for cell death identification. Compared with solution detection, fluorescent imaging provides an effective visualization tool to monitor changes in the distribution and concentration of analytes. In the present study, we developed a cationic cyclometallized iridium(III) complex, $[Ir(pdz)_2(H_2O)_2]^+[OTf]^-$ (**IrC1**, Scheme 1), as a luminescent marker for discriminating dead cells from live cells. **IrC1** displays a red emission band with a maximum wavelength of 625 nm upon excitation at 405 nm. Notably, the **IrC1** complex specifically stains nuclei of dead cells with higher photostability

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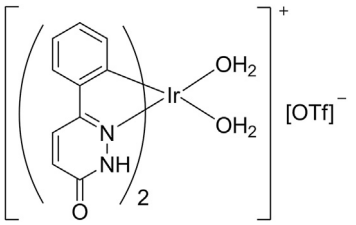

Scheme 1. Chemical structure of iridium(III) complex $[Ir(pdz)_2(H_2O)_2]^+[OTf]^-$(**IrC1**).

and longer lifetime than organic dyes, and facilitates quantitative distinction of early apoptotic from dead cells in combination with flow cytometry.

2. Experimental section

2.1. Materials and general instruments

All starting materials were obtained from commercial supplies and used as received. Phosphate buffered saline (PBS), Fetal bovine serum (FBS), DMSO, L–alanine (Ala), L–arginine (Arg), L–asparagine (Asn), L–aspartic acid (Asp) L–glutamine (Gln), L–glycine (Gly), L–isoleucine (Ile), L–leucine (Leu), L–lysine (Lys), L–phenylalanine (Phe), L–proline (Pro), L–serine (Ser), L–threonine (Thr), L–tryptophan (Try), L–tyrosine (Tyr), L–valine (Val), L–glutamic acid (Glu), L–cysteine (Cys), L–methionine (Met), L–histidine (His), bovine serum albumin (BSA), deoxyribonucleotide triphosphate (dNTP) and CT DNA were obtained from Acros. Pyridazine (pdz) and 2–ethoxyethanol were obtained from J&K. $IrCl_3\cdot 3H_2O$ was an industrial product and used without further purification. Hoechst 33258, propidium iodide (PI), DAPI and Annexin V-FITC reagents were purchased from Invitrogen.

UV–visible spectra were recorded on a Shimadzu UV–2550 spectrometer. Steady–state emission experiments at room temperature and 77 K were measured on an Edinburgh instrument FL–900 spectrometer with Xe lamp as excitation source. Luminescence lifetime studies were performed with an Edinburgh FL–900 photo–counting system with a hydrogen–filled lamp as the excitation source. Luminescence quantum yield (Φ_{em}) of **IrC1** in aerated solution was measured by Hamamatsu quantum yield spectrometer C11347.

2.2. Synthesis of IrC1

The complex **IrC1** was synthesized according to previously reported methods [19]. Briefly, a mixture of 2-ethoxyethanol and water (3:1, v/v) was added to a flask containing $IrC1_3\cdot 3H_2O$ (1.0 mmol) and the derivative ligands of pyridazine (pdz) (2.5 mmol). The mixture was refluxed for 24 h. After cooling, the orange solid precipitate was filtered to give crude cyclometalated Ir(III) chloro-bridged dimmer. The chloro-bridged dimer (0.05 mmol) and AgOTf (0.11 mmol) were placed in the 100 mL round bottomed flask. Then 80 mL solvent ligand (DMSO/MeOH = 40:40) was added, and the resulting slurry was stirred for 24 h. The suspension filtered to remove insoluble inorganic salts, the precipitate was washed three times (2 mL) with MeOH. The filtrate was concentrated to dryness under reduced pressure and dried in vacuum 24 h to afford $[Ir(pdz)_2(H_2O)_2]^+[OTf]^-$ in 5% yield as an orange solid. ^{1}H NMR (DMSO-d6, 400 MHz): δ (ppm) 8.09 (d, J = 9.9 Hz, 1H), 7.92 (d, J = 9.5 Hz, 1H), 7.39 (d, J = 7.4 Hz, 1H), 7.31 (d, J = 7.2 Hz, 1H), 6.98 (d, J = 10.0 Hz, 2H), 6.81 (d, J = 7.3 Hz, 1H), 6.63–6.73 (m, 4H), 5.79 (d, J = 7.3 Hz, 1H). FT-IR: 1637 cm^{-1} (C=O), 842, 776 and 740 cm^{-1} (Ar–H). Exact Mass: Calcd for $C_{20}H_{18}IrN_4O_4$, 571.1. Found: MALDI-TOF: m/z: 570.9 (M+), 535.0 $[M-2H_2O]^+$.

2.3. Cell culture and cytotoxicity

2.3.1. Cell culture

Human epithelial cervical cancer cell line (KB), Human nasopharyngeal carcinoma cell line (HeLa), Human liver hepatocellular carcinoma (HepG2) and Human normal hepatocyte (HL-7702) cell lines were provided by the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (CAS). HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v FBS and 1% antibiotic/antimycotic solution (penicillin and streptomycin, Invitrogen). KB, HepG2 and HL-7702 cells were grown in RPMI 1640 supplemented with 10% v/v FBS and with and 1% antibiotic/antimycotic solution. All cells cultures were kept at 37 °C in a humidified incubator with 5% CO_2.

2.3.2. Cytotoxicity assay of *IrC1*

The *in vitro* cytotoxicity was evaluated by performing the methyl thioazolyl tetrazolium (MTT, Sigma–Aldrich) assay in KB cells. Cells growing in log phase were seeded into 96-well cell-culture plate at 1×10^4/well and then incubated for 24 h at 37 °C under 5% CO_2. The cluster complex **IrC1** (100 μL/well) at different concentrations (20, 30, 40, 50, 60, 70, 80, 90 μM, diluted in RPMI 1640) were added to the wells of the treatment group, and RPMI 1640 to the negative control group. The cells were then incubated for 24 h at 37 °C under 5% CO_2. Thereafter, MTT (20 μL; 5 mg/mL) was added to each well, and the plate was incubated for an additional 4 h at 37 °C under 5% CO_2. After changing the culture medium to 100 μL DMSO, the assay plate was allowed to stand at room temperature for 15 min. An enzyme-linked immunosorbent assay (ELISA) reader (infinite M200, Tecan, Austria) was used to measure the OD570 (Absorbance value) of each well with background subtraction at 690 nm. The following formula was used to calculate the viability of cell growth:

Cell viability (%) = (mean of Absorbance value of treatment group/mean of A value of control) × 100.

2.4. Inducing of cell apoptosis

Hydrogen peroxide (H_2O_2) incubation to generate an intracellular non-reversible oxidative stress and H_2O_2 at low dose triggers the early apoptotic machinery. 35 mm culture dishes with KB cells (in DMEM) were incubated with 3% of hydrogen peroxide for 2 h. After treatment, cells were washed for 3 times in phosphate buffered saline solution (PBS, Gibco) and maintained in the incubator until they were processed for observation.

2.5. Luminescence bioimaging

2.5.1. Live cell imaging

Cells (5×10^8/L) were plated on 14 mm glass coverslips and allowed to adhere for 24 h. The cells were washed with PBS and then incubated solely with 10 μm **IrC1** in DMSO/PBS (pH 7.4, 1:99, v/v) for 10 min at 37 or 4 °C. Cell imaging was then carried out after washing the cells with PBS.

Luminescence imaging including xy-scan, lambda-scan, T-scan, and time-lapse-imaging, was performed with an Olympus FluoView FV 1000 confocal fluorescence microscope and a 60 × oil-immersion objective lens. Cells incubated with **IrC1** were excited at 405 nm with a semiconductor laser, and the emission was collected at 625 ± 20 nm. Quantization by line plots was accomplished using the software package provided by Olympus instruments.

2.5.2. Co-staining of dead cells with *IrC1* and DAPI

The cells were detached from the culture and were fixed with 4% paraformaldehyde at room temperature for 20 min. After washing with PBS, the fixed cells were incubated with 10 μm **IrC1** in DMSO/PBS (pH 7.4, 1:99, v/v) for 10 min at 37 °C, and then further stained with DAPI for another 20 min. After washing with PBS, the coverslips were separated from the chamber, and the cells were mounted with 10% glycerol and sealed with nail varnish on a glass substrate.

Cells incubated with **IrC1** were excited at 405 nm with a semiconductor laser, and the emission was collected at 625 ± 20 nm. Quantization by line plots was accomplished using the software package provided by Olympus instruments. DAPI was excited using a laser at 405 nm, and the emission was collected at 460 ± 20 nm.

2.5.3. Monitoring of *IrC1* in apoptotic cells

For apoptosis imaging experiments, KB cells (5×10^8/L) were grown on coverslips (Fisher Scientific, Atlanta, GA) in RPMI 1640. To induce apoptosis, KB cells were incubated with 3% of hydrogen peroxide for up to 2 h. After incubation with hydrogen peroxide, the cells were washed with PBS and incubated for 10 min at 37 °C with 10 μM **IrC1** and Annexin V-FITC. Then cells were fixed onto the coverslips with 2 wt% formaldehyde. Cells undergoing apoptosis were visualized with the confocal imaging microscope (Olympus FluoView FV 1000) equipped with green fluorescence channel for Annexin V-FITC (λ_{ex} = 488 nm, λ_{em} = 530 ± 20 nm) and red channel for **IrC1** (λ_{ex} = 405 nm, λ_{em} = 625 ± 20 nm).

2.5.4. Live cells incubated with liposomes encapsulated with *IrC1*

The feature of liposomes is the ability to encapsulate a large number of **IrC1** and deliver them into cells. Living cells incubated with **IrC1**-containing liposomes for 10 h at 37 °C were performed by confocal luminescent imaging under the excitation 405 nm and emission at 625 ± 20 nm.

2.6. Fluorescence lifetime imaging microscopy (FLIM)

After incubation of 10 μM **IrC1** and Annexin-FTIC in the dead KB cells, which were fixed by addition of 4% paraformaldehyde/2.5% glutaraldehyde for 1 h. The FLIM image setup is integrated with Olympus IX81 laser scanning confocal microscope. The dead cells attached onto a slide glass were covered with a thin cover glass, on which an excitation beam was focused. The light from the pulse diode laser head (PicoQuant, PDL 800-D) with excitation wavelength of 405 nm and frequency of 0.5 MHz was focused onto the sample with a 40×/NA 0.95 objective for single-photon excitation. The correlative calculation of the data was performed by professional software which was provided by PicoQuant company.

2.7. Quantification detection

2.7.1. Flow cytometry analysis

Cellular uptake of **IrC1** under different conditions was analyzed by flow cytometry (Beckman Counter). Cells were filtered through 41 μM nylon mesh in