Electronic Supplementary Information

Cyclometalated iridium(III) complexes for mitochondria-targeted combined chemo-photodynamic therapy

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Experimental section

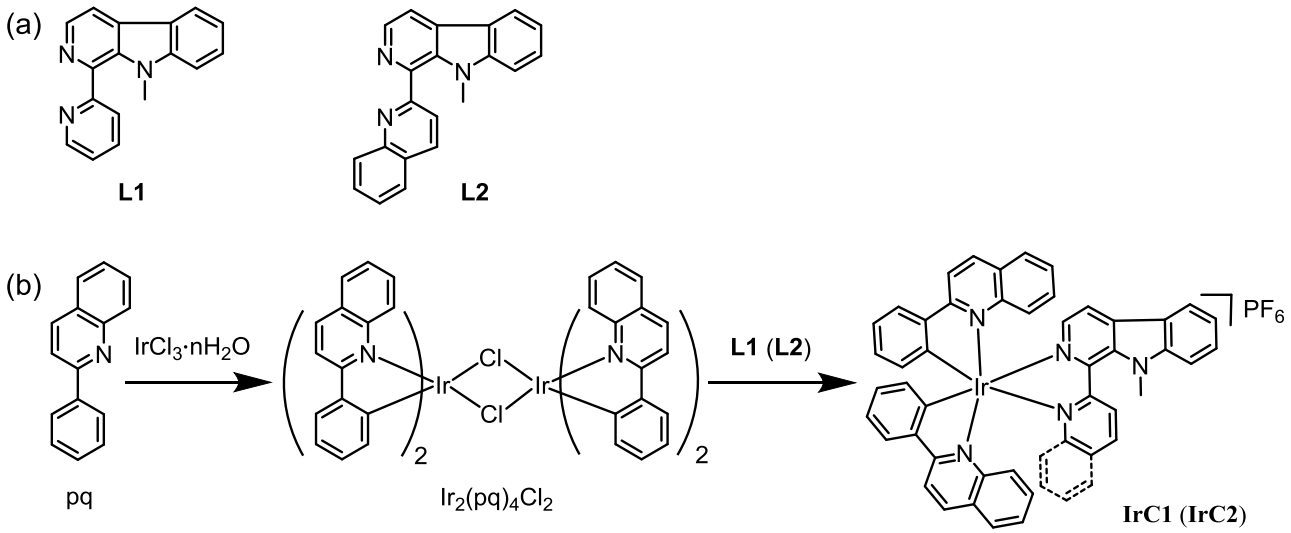
Materials and measurements

Materials. $IrCl_3 \cdot nH_2O$ (Alfa Aesar), 2-phenylquinoline (pq, Sigma Aldrich), NH_4PF_6 (Alfa Aesar), dimethyl sulfoxide (DMSO, Sigma Aldrich), 1,3-diphenylbenzofuran (DPBF, Sigma Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich), cisplatin (Sigma Aldrich), MitoTracker Green FM (MTG, Thermo Fisher Scientific), 2',7'-dichlorofluorescein diacetate (H_2DCF-DA, Sigma Aldrich), MitoSOX-Red (Thermo Fisher Scientific), PicoGreen (Molecular Probes Inc.) and propidium iodide (Sigma Aldrich) were used as received. CellTiter-Glo® Luminescent Cell Viability Assay kit was purchased from Promega. Proteasome 20S Activity Assay Kit and Lipid Peroxidation (MDA) Assay Kit were purchased from Abcam.

Measurements. ESI-MS: LCMS-2010A liquid chromatography-mass spectrometer and Thermo LCQ-DECA-XP liquid chromatography-mass spectrometer; ^{1}H and ^{13}C NMR: Bruker Avance 400 spectrometer; Elemental Analysis (EA): elemental Vario EL elemental analyzer from Germany Element; UV-Vis spectra: American Varian Cary 300 UV/Vis spectrophotometer; Fluorescence spectra and lifetime measurements: Edinburgh FLS920 spectrometer; Microplate reader: iMark absorbance microplate reader (Bio-Rad, USA); Laser confocal fluorescence microscope: Carl Zeiss LSM 710, Germany.

Synthesis and characterization

Ligands **L1** and **L2**, and the dimeric iridium(III) precursor $[Ir_2(pq)_4Cl_2]$ were synthesized according to the methods previously reported.[1]



Scheme S1. (a) Chemical structures of **L1** and **L2**. (b) Synthetic procedures of iridium(III) complexes **IrC1** and **IrC2**.

Crystallographic structure determination[1]

Crystals of **IrC1** qualified for X-ray analysis were obtained by diffusion of diethyl ether to the dichloromethane solution. X-ray diffraction measurements were performed on a Bruker Smart 1000 CCD diffractometer with Mo Kα radiation (λ = 0.71073 Å) at 298 K. The crystal structure of **IrC1** were solved by direct methods with program SHELXS and refined using the full-matrix least-squares program SHELXL.[2] The CCDC deposit numbers, crystallographic data, details of data collection and structure refinements are listed in Table S1. Selected bond distances and angles are listed in Table S2. The structural plots were drawn using the xp package in SHELXTL at a 30% thermal ellipsoids probability level.

Measurement of two-photon absorption (TPA) cross-section

The TPA cross-sections were measured as previously reported.[1] The TP excited emission spectra of **IrC1** and **IrC2** at 730-870 nm were tested in DMSO under the excitation of a nanosecond pulsed laser (OpoletteTM 355II; pulse width ≤ 100 fs; 80 MHz repetition rate; Spectra Physics Inc., USA). Rhodamine B was used as the tandard.[3] By comparing the fluorescence intensity of **IrC1** and **IrC2** with that of Rhodamine B, the TPA cross sections were calculated according to the following equation.[4]

$$\delta_s = \delta_r \frac{\Phi_r c_r I_s n_s}{\Phi_s c_s I_r n_r}$$

Where I is the integrated fluorescence intensity, c is the concentration, n is the refractive index, Φ is the quantum yield, subscript 'r' stands for reference samples, and 's' stands for the samples.

Measurement of singlet oxygen quantum ($^1O_2$) yield

The $^1O_2$ quantum yields (Φ_Δ) for complexes **IrC1** and **IrC2** were evaluated as previously reported.[5] 1,3-diphenylisobenzofuran (DPBF) as the $^1O_2$ indicator and $[Ru(bpy)_3]Cl_2$ as the standard (Φ_Δ = 0.81).[6]

***In vitro* anticancer activities and PDT activities**

The cytotoxicity in the dark and phototoxicity of the tested compounds was determined as previously reported.[5,7] Cell viability was tested by MTT assay. Cells seeded in 96 well plate (1×10^4 cells/160 μL) were incubated overnight, followed by treatment with the tested compounds for 44 h. After adding 20 μL of MTT (5 mg/mL) to each well of the plate, cells were further incubated at 37 ℃ for 4 h. Aspirate the cell medium and add 150 μL of DMSO to each well. The OD value at 595 nm was measured using a microplate reader.

PDT activities: After 12 hours of drug treatment, light irradiation was applied as follows: cells were irradiated with an LED light source (40 mW·cm^{-2}) with a wavelength of 425 nm for 15 min. Subsequent operations were identical to the dark cytotoxicity tests described above.

Colocalization assay[1]

The cells were first stained with MTG (150 nM) for 30 min at 37 ℃, and then further incubated with complexes **IrC1** (1 μM) and **IrC2** (1 μM) for 10 min. After being washed with PBS three times, the cells were visualized by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany) with a 63× oil-immersion objective lens immmediately. The wavelengths for one- and two-photon excitation of Ir(III) complexes are 405 nm and 810 nm, respectively. The excitation wavelength of MTG is 488 nm. Emission was collected at 550−600 nm for **IrC1**, 600−650 nm for **IrC2**, and 500−530 nm for MTG.

ROS and superoxide detection[1]

A549 cells seeded in 35-mm tissue culture dishes were incubated overnight, followed by treatment with treated with **IrC1** (2 μM) at 37 ºC for 1 h. After spirating the old cell medium, fresh serum-free RPMI 1640 containing 10 μM H_2DCF-DA was added and the cells were incubated at 37 ℃ in the dark for 20 min. The cell culture medium was replaced with PBS. In the case of light conditions, the cells were subjected to one-photon (425 nm, 40 mW cm^{-2}) and two-photon (810 nm, 100 mW, 80 MHz, 100 fs) light irradiation for 30 s. The cells were then visualized immediately by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). λ_{ex} = 488 nm and λ_{em} = 520 ± 20 nm for DCF. For superoxide detection, cells were loaded with 5 μM MitoSOX-Red for 10 min in the dark, and the subsequent operations were identical to ROS detection described above. λ_{ex} = 514 nm and λ_{em} = 580 ± 20 nm for MitoSOX-Red.

Intracellular ATP detection[1]

The assay was performed according to the method previously reported.[1] ATP level was measured using CellTiter-Glo® Luminescent Cell Viability Assay kit (G7570, Promega, USA) according to the manufacturer's instructions. In the case of light conditions, after 1 h **IrC1** treatment, cells were irradiated with light (425 nm, 40 mW cm^{-2}) for 1 min, and then further incubated 37 ℃ for 5 h. Note that a relatively high light dose (36 J cm^{-2}) was used to test the phototoxicity in order to fully investigate the PDT potential of **IrC1** and **IrC2**. In the next few experiments, including ATP detection, mitochondrial DNA damage detection, lipid peroxidation levels and proteasomal activity detection, a 1/15 of the light dose (2.4 J cm^{-2}) was applied due to the following reasons: In these experiments, complex **IrC1** showed a low or moderate effect in the absence of light, reflecting the effect of chemotherapy. When under the condition of light irradiation, scuh dose of light (2.4 J cm^{-2}) is enough to improve the effects obviously, reflecting the PDT effect. After optimizing the experimental conditions, such illumination conditions are finally selected.

Mitochondrial DNA damage (PicoGreen staining)

A549 cells seeded in 35 mm tissue culture dishes (Corning, USA) were pretreated with the PicoGreen reagent (3 μL/mL) in growth media at 37 ℃ for 1 h. After that, the cell media were replaced with fresh media containing 1 μM of **IrC1**, and the cells were further incubated at 37 ℃ for

6 h. In the case of light conditions, after 1 h **IrC1** treatment, cells were irradiated with light (425 nm, 40 mW cm^{-2}) for 1 min, and then further incubated 37 ℃ for 5 h. The cells were washed twice with PBS and visualized immediately by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). Emission was collected at 520 ± 20 nm upon excitation at 488 nm.

Lipid peroxidation

Lipid peroxidation was measured by measuring the amount of malondialdehyde (MDA) using a Lipid Peroxidation (MDA) Assay Kit (Abcam, UK) according to the the manufacturer's instructions. In the case of light conditions, after 1 h **IrC1** treatment, cells were irradiated with light (425 nm, 40 mW cm^{-2}) for 1 min, and then further incubated 37 ℃ for 5 h.

Proteasomal activity detection

The assay was performed according to the method previously reported.[1] The assay was performed using Proteasome 20S Activity Assay Kit (Abcam, UK) according to the manufacturer's instructions. In the case of light conditions, after 1 h drug treatment, cells were irradiated with light (425 nm, 40 mW cm^{-2}) for 1 min, and then further incubated 37 ℃ for 5 h.

PDT-induced cell death detection

A549 cells seeded in 35 mm tissue culture dishes (Corning, USA) were pretreated with **IrC1** (1 μM) in cell media at 37 ℃ for 1 h, followed by staining with propidium iodide for 15 min in the dark. The cells were subjected to one-photon (425 nm, 40 mW cm^{-2}) and two-photon (810 nm, 100 mW, 80 MHz, 100 fs) light irradiation for 1 min. After 1 h incubation, the cells were visualized by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). λ_{ex} = 488 nm and λ_{em} = 620 ± 20 nm for propidium iodide.

Supporting figures and tables

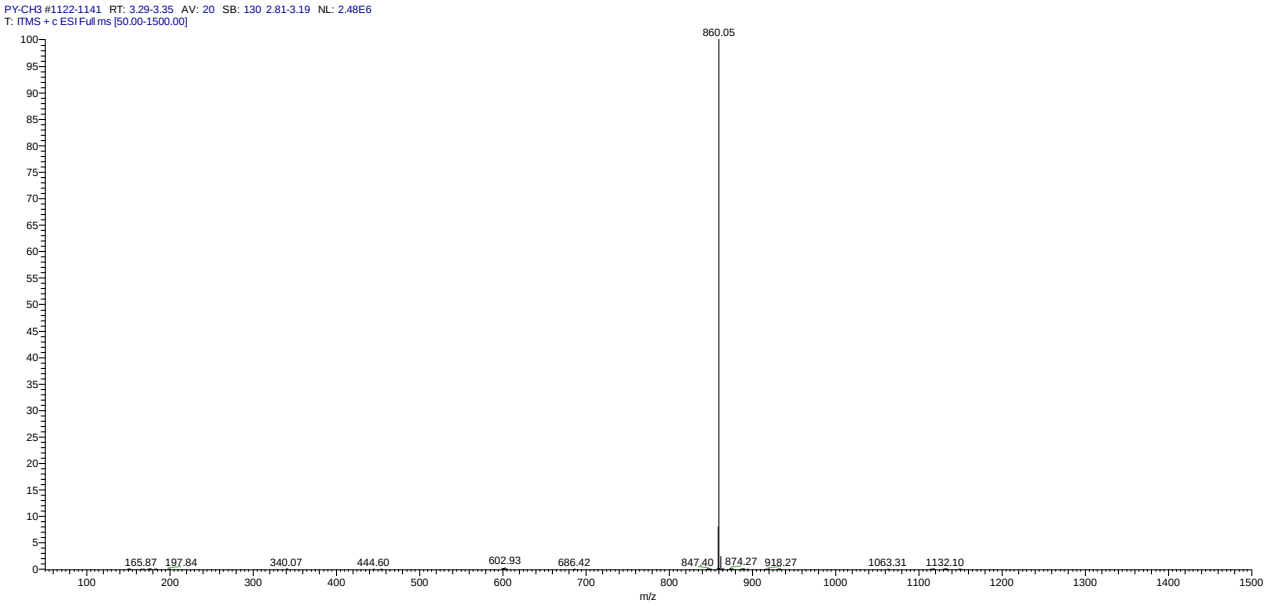


Fig. S1 ESI-MS characterization of **IrC1**, 860.05 $[M-PF_6]^+$.

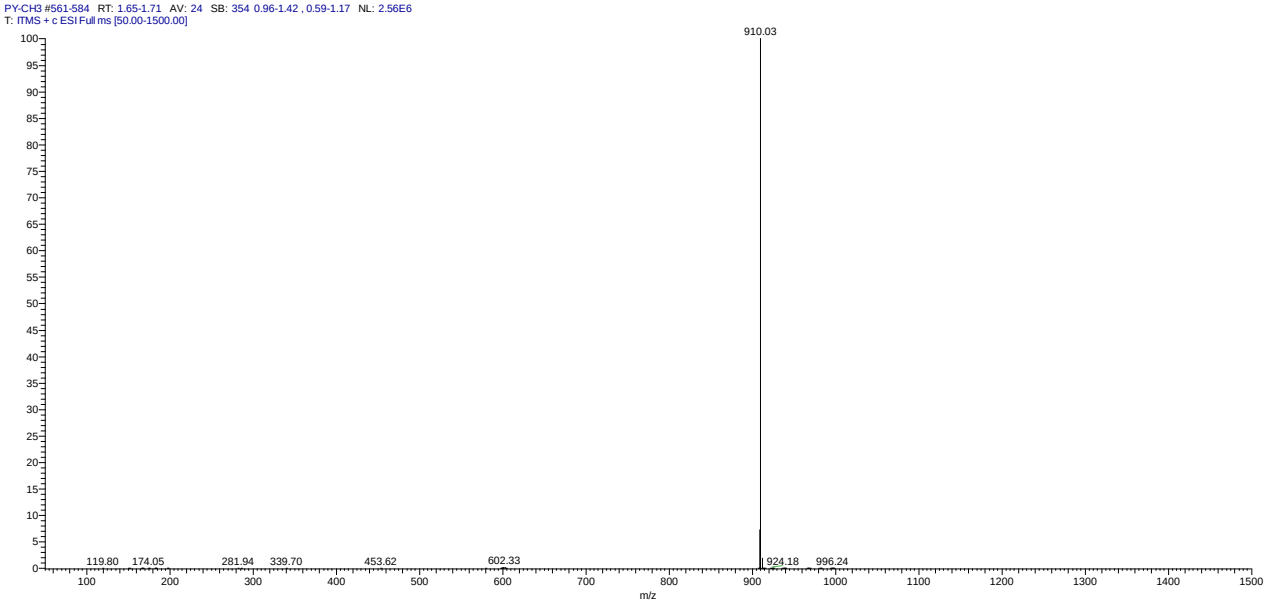


Fig. S2 ESI-MS characterization of **IrC2**, 910.03 $[M-PF_6]^+$.

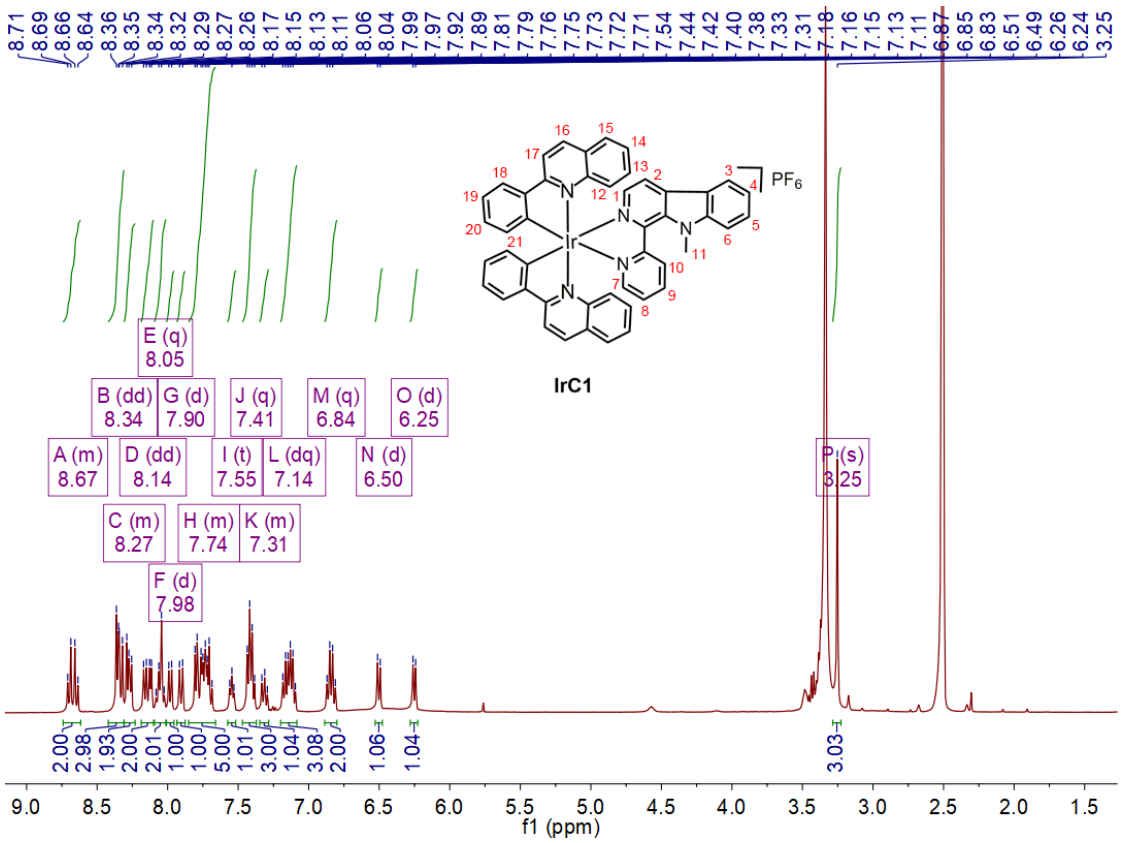


Fig. S3 ^{1}H NMR spectrum of **IrC1** in d_6-DMSO.

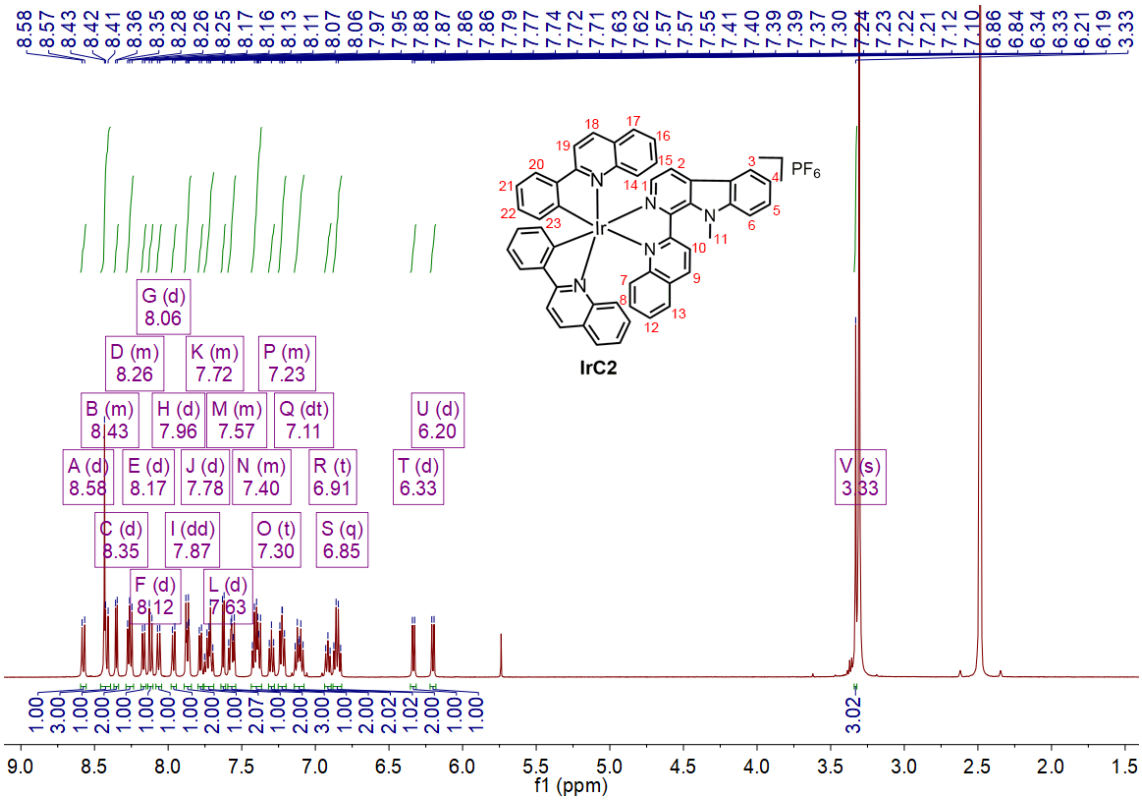


Fig. S4 ^{1}H NMR spectrum of **IrC2** in d_6-DMSO.

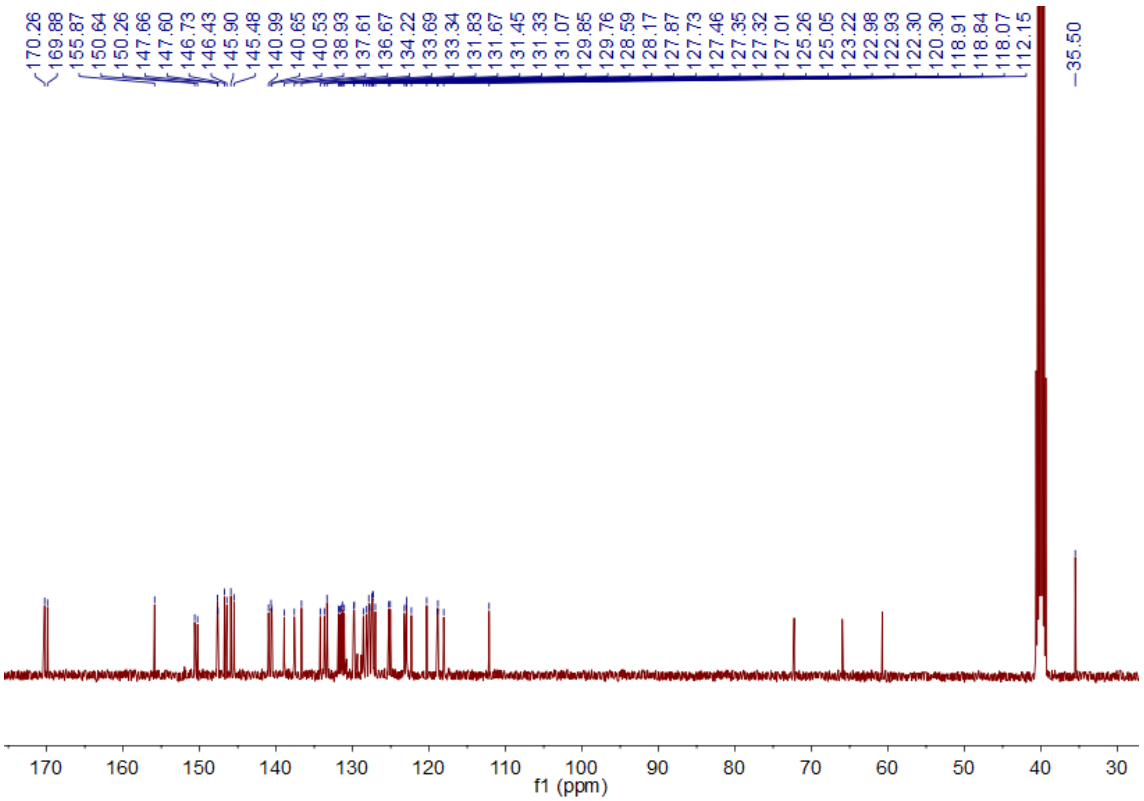


Fig. S5 ^{13}C NMR spectrum of **IrC1** in d_6-DMSO.

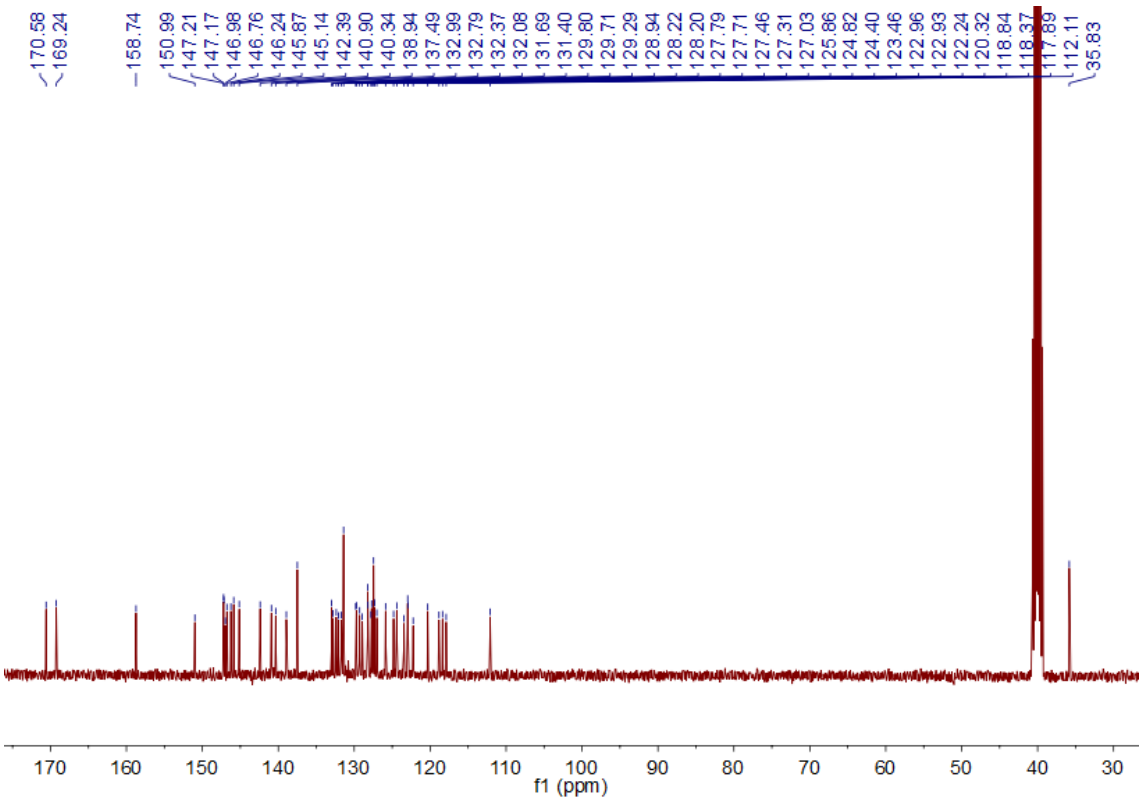


Fig. S6 ^{13}C NMR spectrum of **IrC2** in d_6-DMSO.

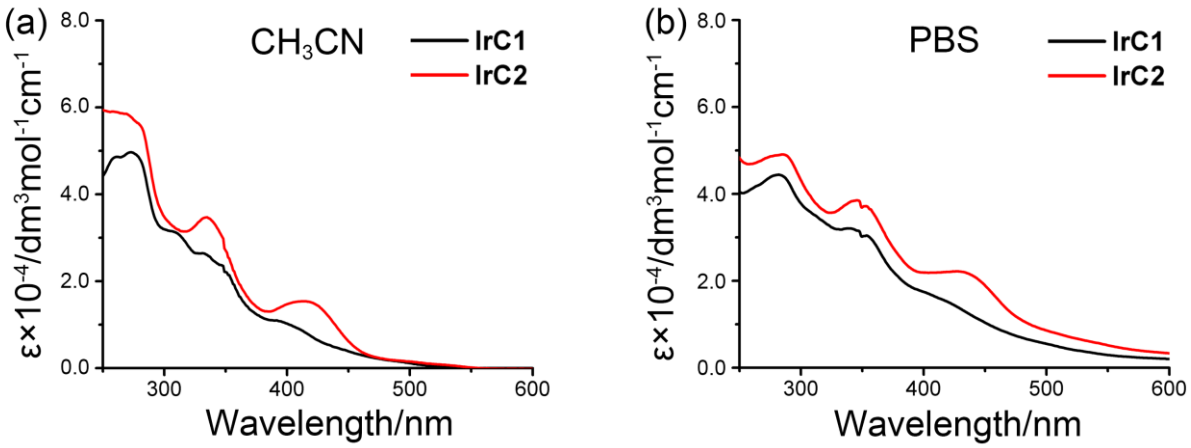


Fig. S7 UV/Vis spectra of **IrC1** and **IrC2** (10 μM) measured in (a) CH_3CN and (b) PBS at 25 °C.

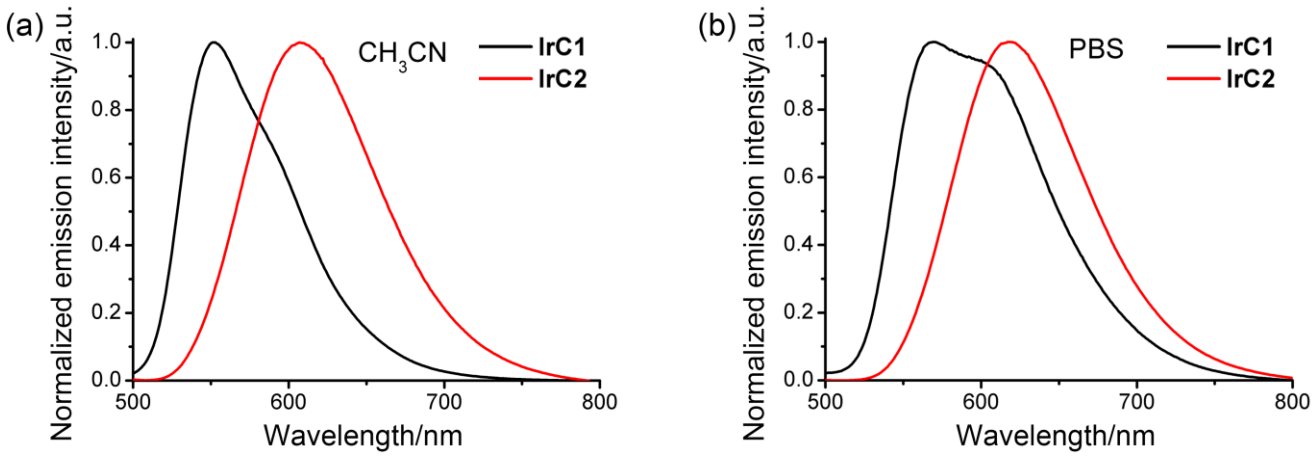


Fig. S8 Emission spectra of complexes **IrC1** and **IrC2** (10 μM) measured in (a) CH_3CN and (b) PBS at 25 °C. λ_{ex} = 405 nm.

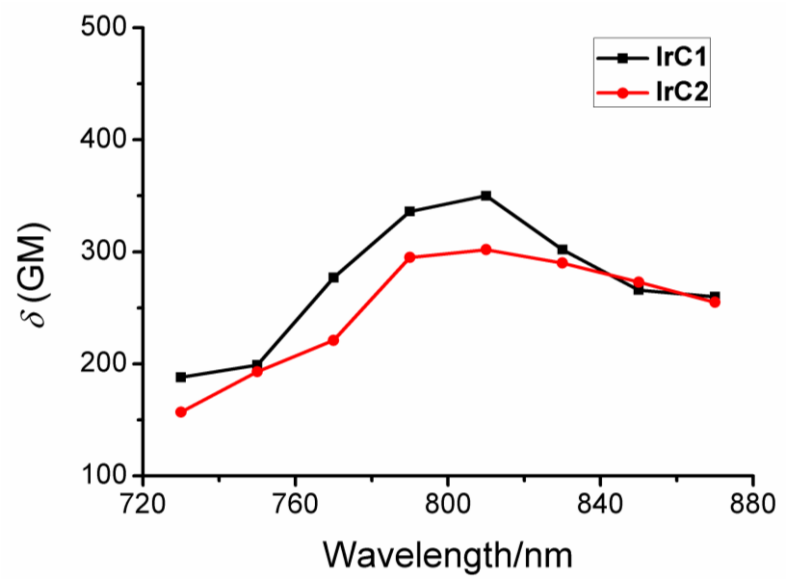


Fig. S9 TPA cross-sections (δ) of **IrC1** and **IrC2** at excitation wavelengths between 730 and 870 nm.

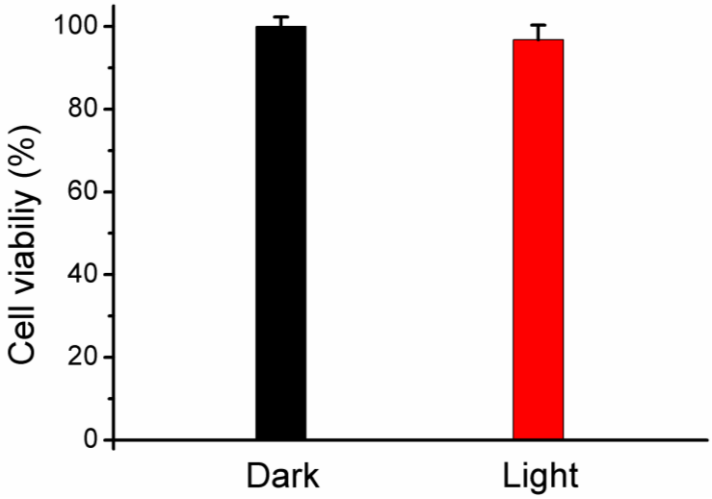


Fig. S10 The effect of light (425 nm, 36 J·cm^{-2}) on the cell viability of untreated A549 cells.

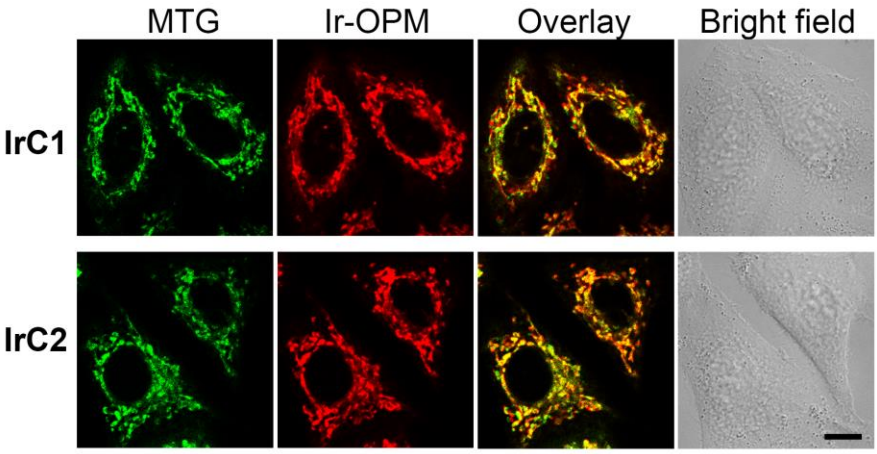


Fig. S11 One-photon excited CLSM images of A549 cells stained with 150 nM MitoTracker Green (MTG) for 30 min, followed by treating with 1 μM of **IrC1** or **IrC2** for 10 min at 37 °C. λ_{ex} = 488 nm and λ_{em} = 500−530 nm for MTG; λ_{ex} = 405 nm and λ_{em} = 550−600 nm for **IrC1**; λ_{ex} = 405 nm and λ_{em} = 600−650 nm for **IrC2**. All of the images share the same scale bar, 10 μm.

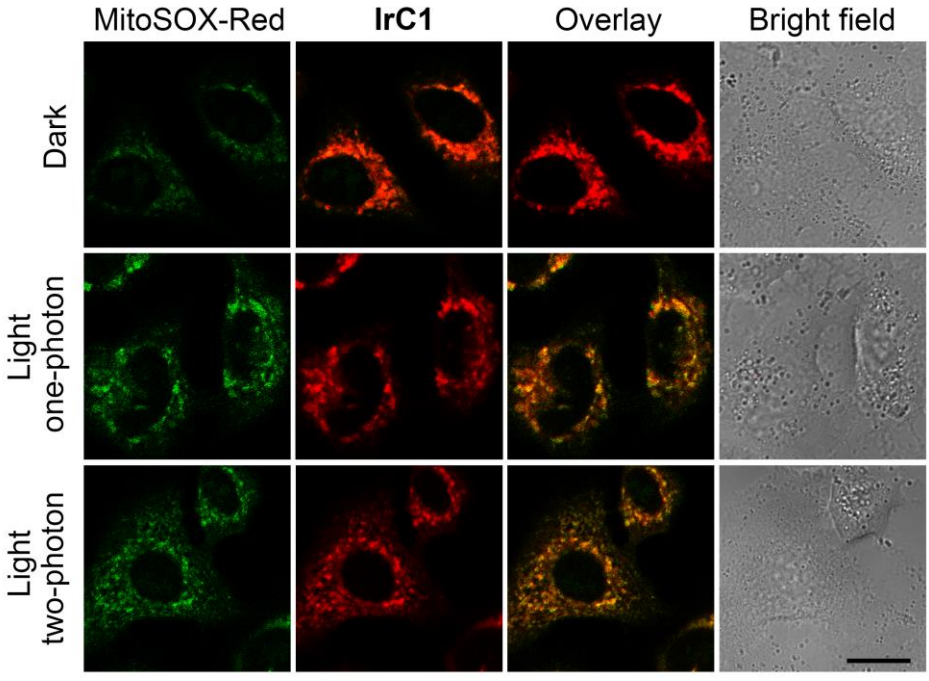


Fig. S12 Detection of intracellular superoxide anion by confocal microscopy after A549 cells were treated with 1 μ M **IrC1** for 1 h and then stained with MitoSOX-Red (5 μM) for 10 min. One-photon light irradiation: 425 nm LED light source; two-photon light irradiation: 810 nm fs laser. All images share the same scale bar, 20 μm.

Table S1 Crystallographic data of **IrC1**

Complex	**IrC1**
CCDC no.	1894673
Empirical formula	$C_{47}H_{32}N_5F_6PIr$
Molecular weight	1003.95
Description	Block, red
Temperature (K)	298 K
λ (Å)	0.71073
Crystal system	monoclinic
Space group	$P2_1/c$
a (Å)	12.4372(13)
b (Å)	15.3865(16)
c (Å)	24.393(2)
α (^{o})	90.00
β (^{o})	102.838(3)
γ (^{o})	90.00
Volume, Å^3	4551.3(8)
Z	4
μ/mm^{-1}	3.030
F(000)	1980.0
θ_{max} (deg)	27.480
Completeness to θ_{max}	0.993
ρ_{calc} (g/cm^3)	1.465
[R_{int}]	0.1476
R1[a][I > 2σ(I)]	0.0935
wR2[a]	0.2840
GOF[b]	0.981

[a] $R1 = \sum\|F_0\| - \|F_c\| / \sum|F_0|, wR2 = \left\{\sum\left[w\left(F_0^2 - F_c^2\right)^2\right] / \sum\left[w\left(F_0^2\right)^2\right]\right\}^{1/2}$ [b] $GOF = \left\{\sum\left[w\left(F_0^2 - F_c^2\right)^2 / (n-p)\right]\right\}^{1/2}$

where *n* is the number of data and *p* is the number of parameters refined.

Table S2 Selected bond lengths (Å) and bond angles (deg) of **IrC1**

Complex	**IrC1**	
bond lengths (Å)	Ir1–N1	2.103(7)
	Ir1–N2	2.132(7)
	Ir1–N3	2.153(9)
	Ir1–N4	2.183(7)
	Ir1–C15	1.972(11)
	Ir1–C30	2.011(10)
bond angles (deg)	N1–Ir1–N2	170.8(3)
	N1–Ir1–N4	81.6(2)
	N3–Ir1–N4	75.1(3)
	C15–Ir1–N1	79.4(3)
	C30–Ir1–N2	79.3(3)
	C15– Ir1–C30	88.5(4)

Table S3 Photophysical data of complexes **IrC1** and **IrC2**

Complex	Medium	λ_{em}[a]/nm	Φ[b]	τ[c]/ns
IrC1	CH_2Cl_2	557	0.368	268
	CH_3CN	559	0.181	170
	PBS	570	0.008	226
IrC2	CH_2Cl_2	611	0.149	152
	CH_3CN	614	0.093	159
	PBS	618	0.022	390

[a] Emission maximum, λ_{ex} = 405 nm. [b] The emission quantum yields were determined using $[Ru(bpy)_3]Cl_2$ in N_2-saturated CH_2Cl_2 (Φ = 0.059),[8] CH_3CN (Φ = 0.062)[9] and PBS (Φ = 0.042)[10] as the references. [c] The lifetimes were measured at the emission maxima.

Table S4 IC_{50} values of tested compounds toward several cells in the dark

Compound	IC_{50} (μM)			
	A549R	HeLa	MCF-7	HLF
IrC1	0.52 ± 0.08	0.98 ± 0.1	1.0 ± 0.1	14.2 ± 2.6
IrC2	0.97 ± 0.2	1.2 ± 0.3	0.92 ± 0.2	15.8 ± 2.3
L1	>100	>100	>100	>100
L2	>50	>50	>50	>50
$Ir_2(pq)_4Cl_2$	18.9 ± 2.2	26.4 ± 3.8	21.7 ± 1.5	47.3 ± 3.8
cisplatin	50.6 ± 6.8	9.4 ± 1.5	12.2 ± 1.1	26.3 ± 3.4

Supporting references

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