Electronic Supporting Information

Theranostic TEMPO-functionalized Ru(II) complexes as photosensitizers and oxidative stress indicators

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Supporting Methods

Quantification of singlet oxygen generation

The $^1O_2$ quantum yields (Φ_Δ) of Ru(Ⅱ) complexes were detected according to the literature procedure[1] with slight modifications. The DMSO solutions containing **1**, **1a**, **2**, **2a** and DPBF (50 μM) were aerated for 10 min, then photo irradiated at 450 (25W·cm^{-2}). Every 2 s, the absorbance of DPBF at 418 nm was recorded. MB was used as the reference (Φ_Δ = 0.52). The absorbance at 450 nm of Ru(Ⅱ)complexes and MB was kept at 0.15. The $^1O_2$ quantum yields of Ru complexes were calculated according the following equation:

$$\Phi\Delta(\mathrm{x}) = \Phi\Delta(std) \times (\frac{S_x}{S_{std}}) \times (\frac{F_{std}}{F_x})$$

where S is the slope of a linear fit of the change of absorbance at 418 nm against the irradiation time(s) and F is the absorption correction factor, which is given by F = $1 - 10^{-OD}$ (OD is the optical density at the irradiation wavelength).

Cell lines and culture conditions

HeLa, A549, A549R and LO2 cells were obtained from Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). The Cells were maintained in DMEM (Dulbecco's modified Eagle'smedium, Gibco BRL) or RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) medium containing 10% FBS (fetal bovine serum, Gibco BRL), 100 μg/mL streptomycin, and 100 U/mL penicillin (Gibco BRL). The cells were cultured in a humidified incubator at 37 °C under 5% CO_2. A549R cells were cultured in a medium containing increasing concentrations of cisplatin to maintain the resistance. In each experiment, cells treated with DMSO (1%, v/v) only were used as the reference group .

Cellular uptake studies

Confocal microscopy

HeLa cells were seeded into 35 mm dishes (Corning) for confocal microscopy. The cells were incubated with **1**, **1a**, **2**, **2a** for 12 h or 24 h and washed twice with cold PBS, then viewed immediately by a confocal laser-scanning microscope by excitation at 488 nm or 800 nm. The emission filters used are 620 ± 20 nm.

ICP-MS

Hela cells were seeded in 10 cm tissue culture dishes and incubated for 24 h. The medium was removed and replaced with fresh medium containing the tested complexes (15 μM). The cells were washed with PBS, trypsinized and collected after 24 h incubation. The cells were counted, and digested with HNO_3 (65%, 2 mL) at 60 °C for 1 h. The solution was then diluted to a final volume of 10 mL with Milli-Q water. The concentration of ruthenium was measured using the XSERIES 2 ICP-MS.

Cytotoxicity Assay[2]

The dark cytotoxicity and phototoxicity of the tested compounds toward Hela, A549, A549cisR, and LO2 cell lines was determined by MTT assay.

Dark cytotoxicity

Cells cultured in 96-well plates were grown to confluence. The compounds were dissolved in DMSO (1%, v/v), and diluted with fresh media immediately. The cells were incubated with a series of concentrations of the tested compounds for 44 h at 37 °C. 20 μL MTT solution then added to each well, and the plates were incubated for an additional 4 h. The media was removed, and DMSO was added (150 μL per well) and incubated for 10 min with shaking. The absorbance at 595 nm was measured using a microplate reader (Infinite M200 Pro, Tecan, Männedorf, Switzerland).

Photo-toxicity

Cells were incubated with the tested compounds for 24 h. The media containing the Ru complexes was removed and fresh media without the complexes was added. Irradiated with a 450 nm LED light array (25 mW cm^{-2}) for 5min (7.5 J cm^{-2}), incubated for another 20 h, MTT was added and incubated for an additional 4 h. For Concentration-dependent inhibition of NaN3 (5 and 10 mM) on cell death assay, complex **1** was incubated with Hela for 24h, media was removed and fresh media without the complexes was added. NAC was added 1h before irradiation, cell was incubated for another 19 h, the cytotoxicity was determined as described above.

The cytotoxicity was determined as described above. No statistical difference in viability was observed between cells kept in the dark and cells irradiated.

Cell viability of cell co-incubated with H_2O_2

Cells cultured in 96-well plates were grown to confluence, complex **1** (2 and 5 μM) was added into cell and incubated for 24h. Then, H_2O_2 diluted with PBS buffer was added to induce oxidative stress, the cell were incubated for 30 min and then washed twice with PBS, 20 μL MTT solution then added to each well. The cytotoxicity was determined as described above.

Detection of apoptosis

Hoechst staining

HeLa cells were seeded into 35 mm dishes (Corning) and incubated for 24 h and then treated with complex 1 for 24 h. Photo-irradiation (450 nm, 25 mW·cm^{-2}, 5 min) was

performed and the cells were then washed twice with cold PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. After washed with cold PBS, cells were labeled with Hoechst 33342 (5 μg/mL in PBS) for 5 min. The cells were analyzed immediately with a confocal laser-scanning microscope.

Annexin V-FITC staining

In flow cytometry, HeLa cells were cultured in 6-well tissue culture plates for 24 h and then treated with complex 1 (1 μM) for 24 h. PDT treatment was carried out as described in Hoechst Staining. The cells were harvested and stained using an annexin V-FITC apoptosis detection kit (Sigma Aldrich) according to the manufacturer's instructions. Data were collected by flow cytometer and analyzed with FlowJo 7.6 software . In confocal microscopy, cells were washed twice with ice-cold PBS, and stained with FITC-labelled annexin at 37 °C for 15 min in the dark, then visualized immediately by confocal microscopy

Measurement of Intracellular ROS (Flow cytometry)

HeLa cells were seeded into 6-well tissue culture plates (Corning) for 24 h and then treated a with complex **1** or **1a** for 24 h, followed by the irradiation (450 nm, 25 mW·cm-2, 5 min). After washed twice with serum-free DMEM, the fluorescence intensity of cells was measured immediately by flow cytometry with excitation at 488 nm and emission at 530 nm. Green mean fluorescence intensities were analyzed using FlowJo 7.6 software.

The effect of 1-1a on Hela Cells against H_2O_2-induced oxidative damage was also checked by flow cytometry. After incubation with complex 1 or 1a for 24 h at the indicated concentrations for 24 h, 2 mM H_2O_2 was added into each wells and incubated for another 30 min at 37 °C and 5% CO_2. Then H_2O_2 was carefully removed and the cell was washed with PBS twice immediately before further characterization by flow cytometry.

Monitoring the intracellular Fluorescence Intensity of 1 & 1a

Flow cytometry

HeLa cells were seeded into 6-well tissue culture plates (Corning) for 24 h and then incubated with complex 1 or 1a at the indicated concentrations for 24 h in the dark, followed by another incubation with light irradiation (25 mW cm^{-2}, 5 min) or H_2O_2 co-incubation (2 mM, 30 min). After washed twice with PBS, the cells were harvested and the fluorescence intensity of cells was measured immediately by flow cytometry with excitation at 488 nm and emission at 585 ± 21 nm.

Confocal microscopy

HeLa cells were seeded into 35 mm dishes (Corning) and incubated for 24 h and then treated with complex **1** (10 μM) for 24 h. Light irradiation and H_2O_2 co-incubation were carried out as described above. The cells were washed twice with ice-cold PBS, then visualized immediately by confocal microscopy.

Statistical analysis

All biological experiments were performed at least twice with triplicates in each experiment. Representative results were depicted in this report and data were presented as means ± standard deviations.

Supporting Figures and Tables

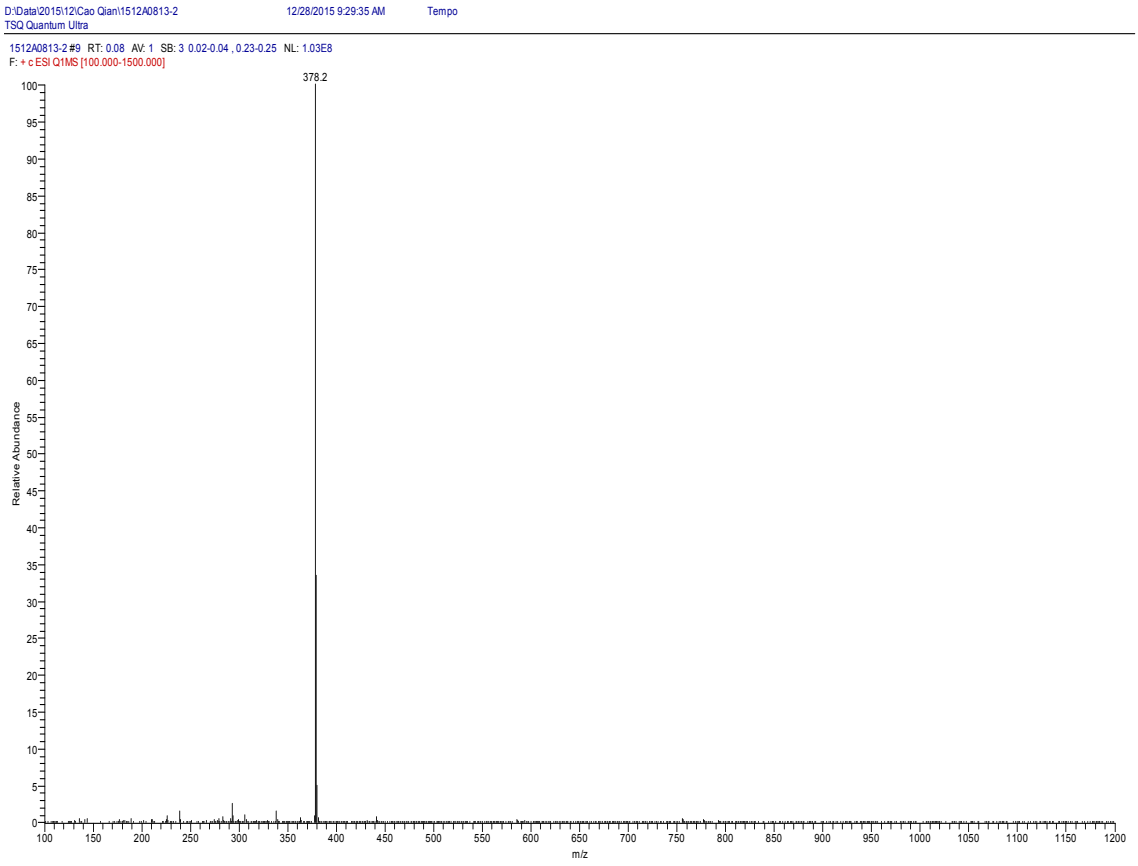

Fig. S1 (a) ESI-MS spectrum of ligand **L**, 378.2, $[M+H]^+$; Elemental analysis: Calcd(%) for $C_{22}H_{25}N_4O_2$ •$1CH_3CN$ • $1H_2O$: C, 66.03; H, 6.93; N, 16.04; C/N, 4.117; C/H, 9.528 ; Found: C, 66.07; H, 6.955; N, 15.99; C/N,4.131; C/H, 9.499 ;

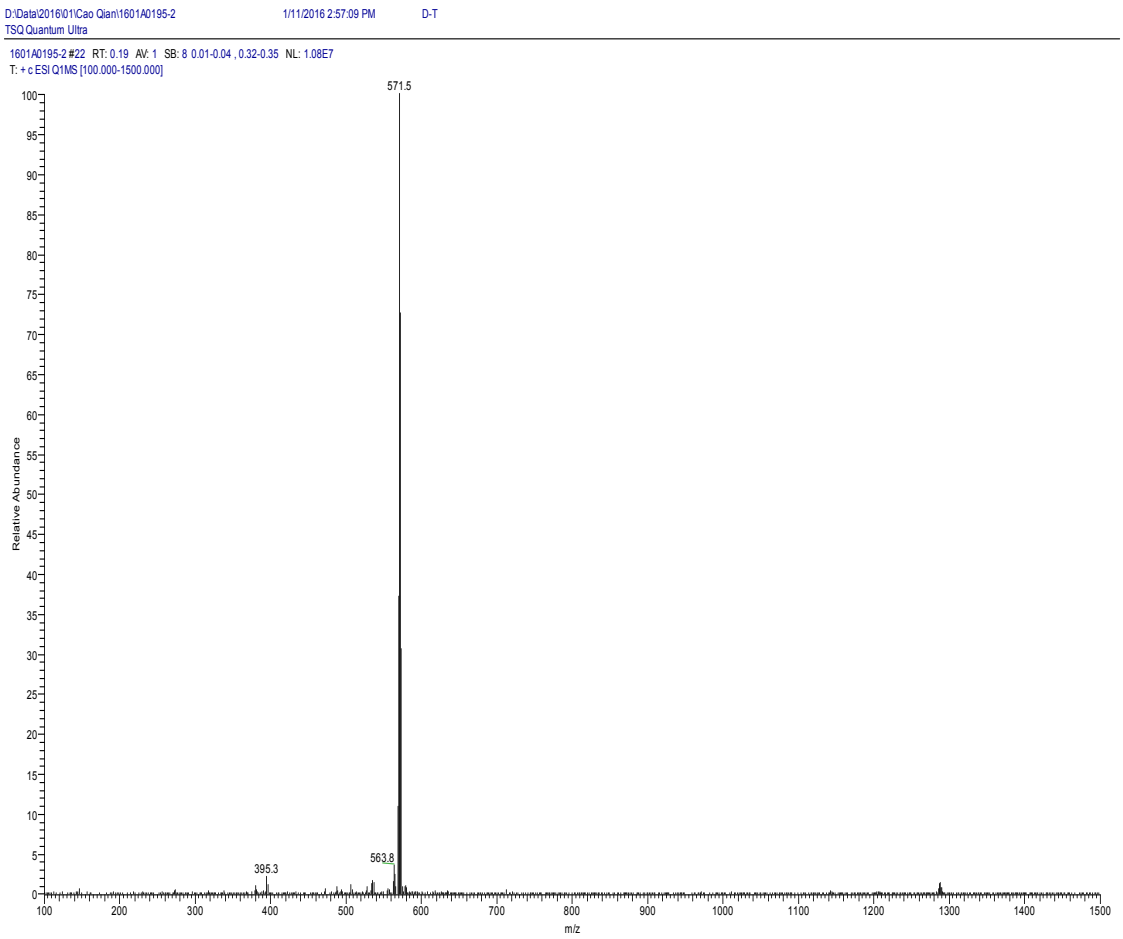

Fig. S1 (b) ESI-MS spectrum of complex **1**, 571.5 , 1/2$[M+H]^{2+}$; Elemental analysis calcd(%) for $C_{74}H_{71}F_{12}N_8O_5P_2Ru$ •$2CH_3OCH_3$ • $1H_2O$: C, 57.59; H, 4.64; N, 7.26; C/N, 7.932; C/H, 12.412 ; found: C, 57.53; H, 4.68; N, 7.05; *C/N*,8.160;C/H, 12.292;

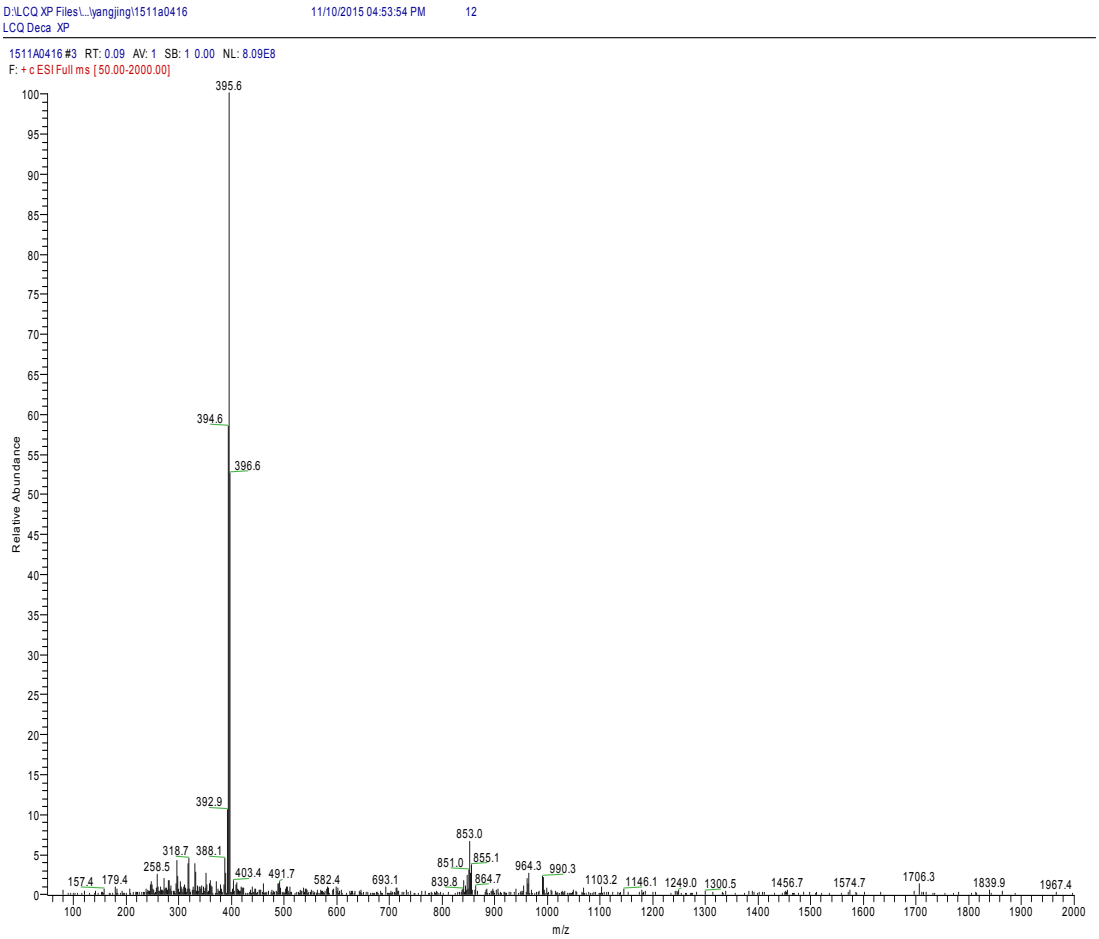

Fig. S1 (c) ESI-MS spectrum of complex **2**, 395.6, 1/2[M+H]$^{2+}$; Elemental analysis calcd(%) for $C_{42}H_{41}F_{12}N_8O_2P_2Ru$ • $1CH_3OCH_3$ • $1H_2O$: C, 46.16; H, 4.31; N, 9.79; C/N, 4.715; C/H, 10.709; found: C, 46.24; H, 4.35; N, 9.81; C/N, 4.713; C/H, 10.629.

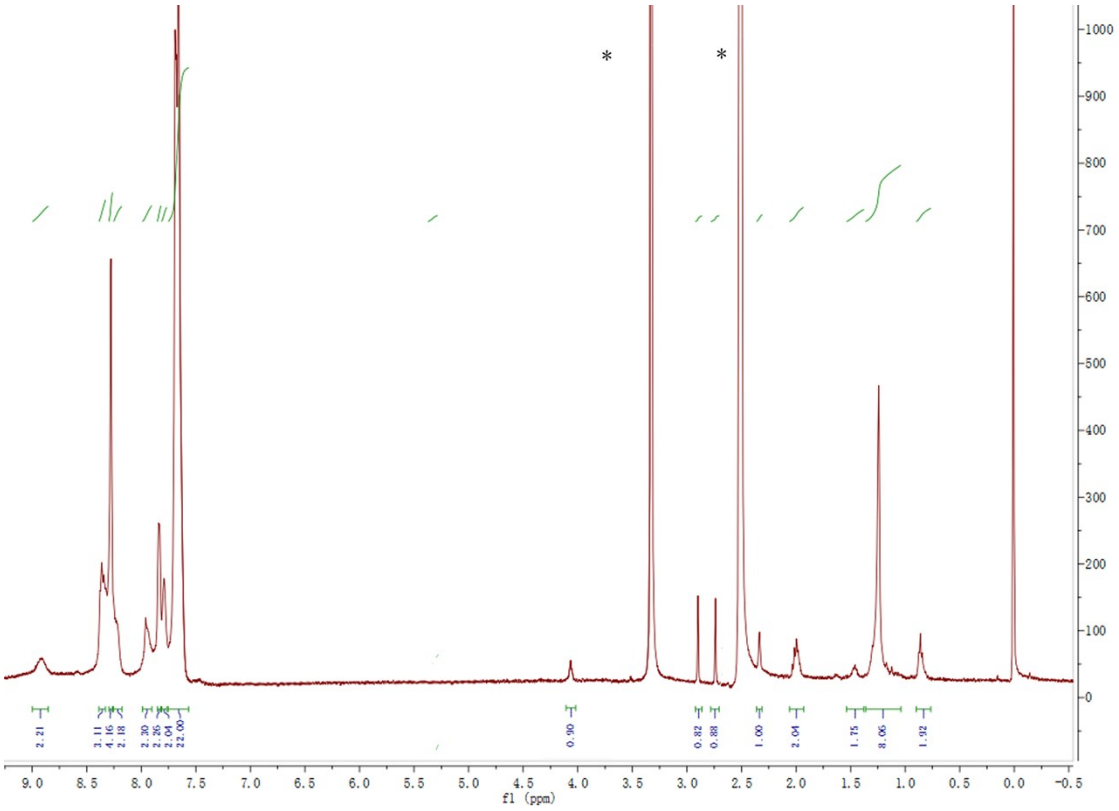

Fig. S1 (d) 400 MHz ^{1}H NMR spectrum of complex **1** in dimethyl sulfoxide-d6 (*corresponds to residual solvents peaks of H_2O and dimethyl sulfoxide-d6).

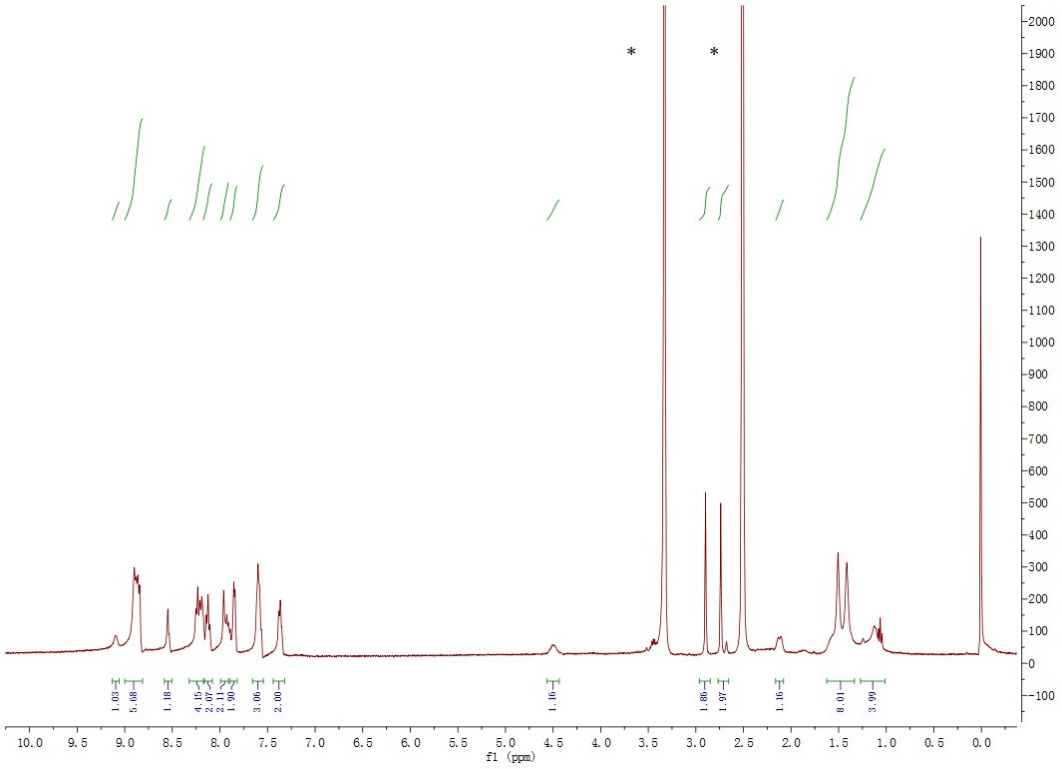

Fig. S1 (e) 400 MHz ^{1}H NMR spectrum of complex **2** in dimethyl sulfoxide-d6 (*corresponds to residual solvents peaks of H_2O and dimethyl sulfoxide-d6).

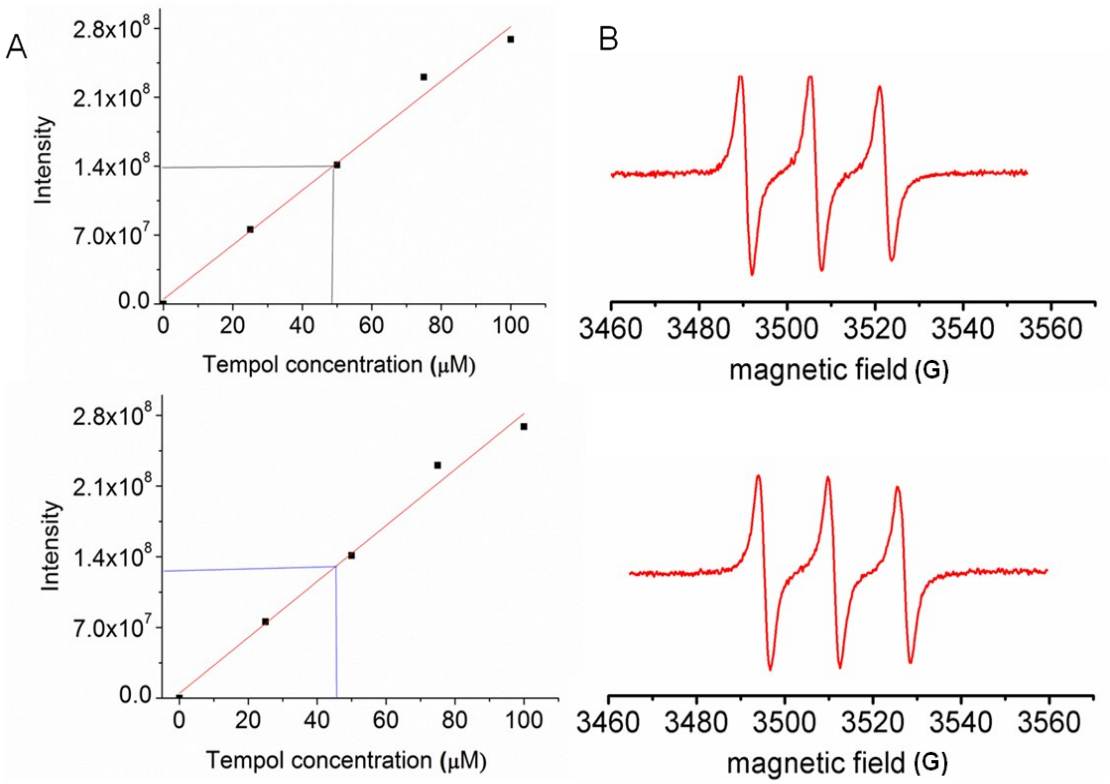

Fig. S2 (A) ESR standard curve showed the intensity of the ESR signal as a function of the TEMPO concentration, accordingly determined the spin efficiency of complex **1-2**. (B) ESR spectra of 5×10^{-4} M of complex **1-2** in acetonitrile. Accordingly, effective spin concentration of complex **1** and **2** was determined to be 98% and 95%, respectively.

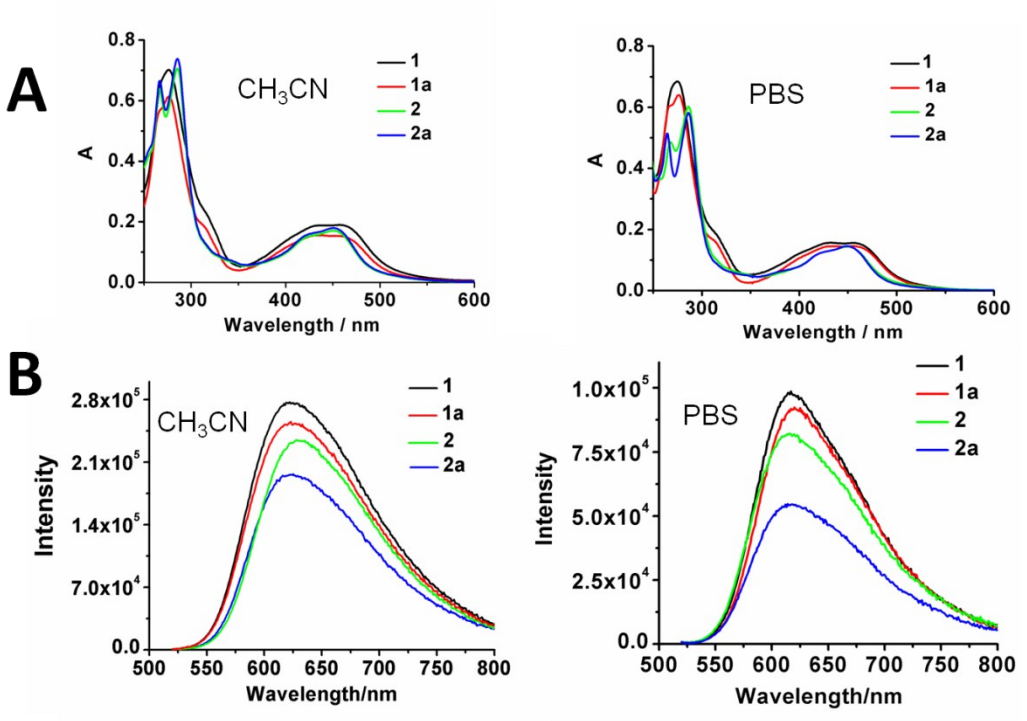

Fig. S3 (A)UV/Vis and (B) emission spectra of complexes **1, 1a, 2, 2a (**10 μM) measured in CH_3CN and PBS at 298 K. (excitation at 450 nm).

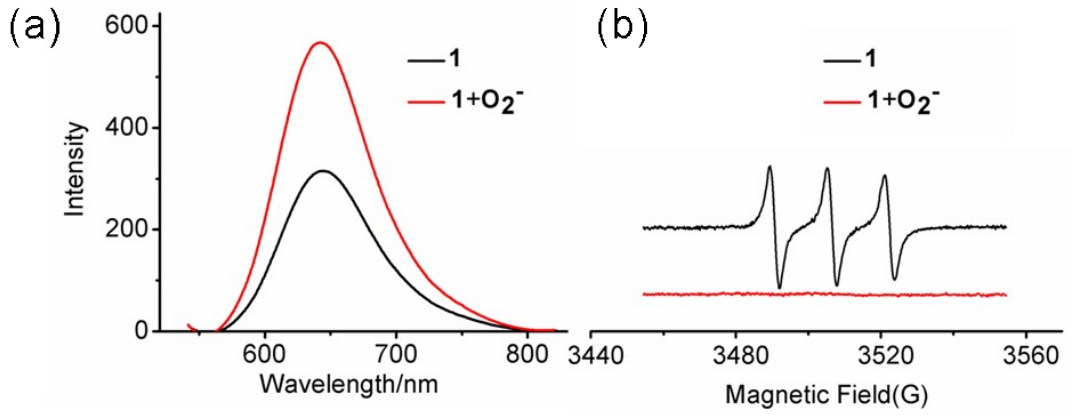

Fig. S4 (a)Emission and (b) ESR spectra of **1** (30 μM) recorded in phosphate buffer pH 8.2 in the presence of Xanthien(100uM)/Xanthine oxidase (100mU/ml) system which is the source of $O_2^{\bullet-}$.

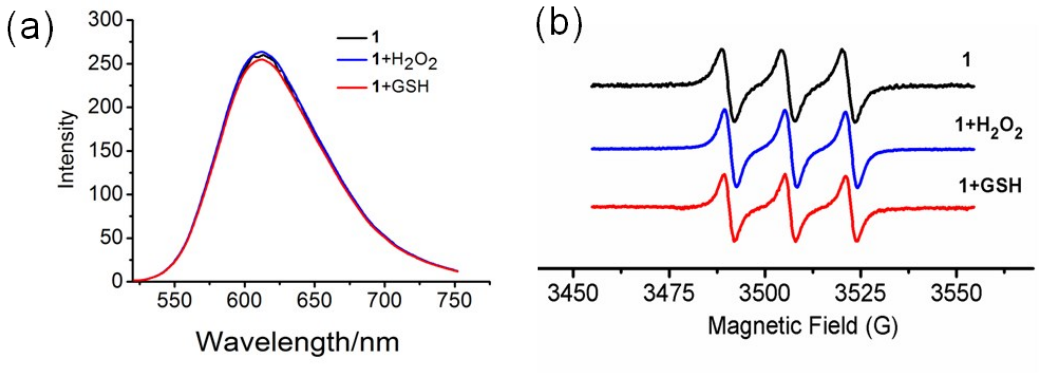

Fig. S5 (a)Emission and (b) ESR spectra of **1** (30 μM) recorded in phosphate buffer pH7.5 in the presence of H_2O_2 or GSH (3×10^{-3} M).

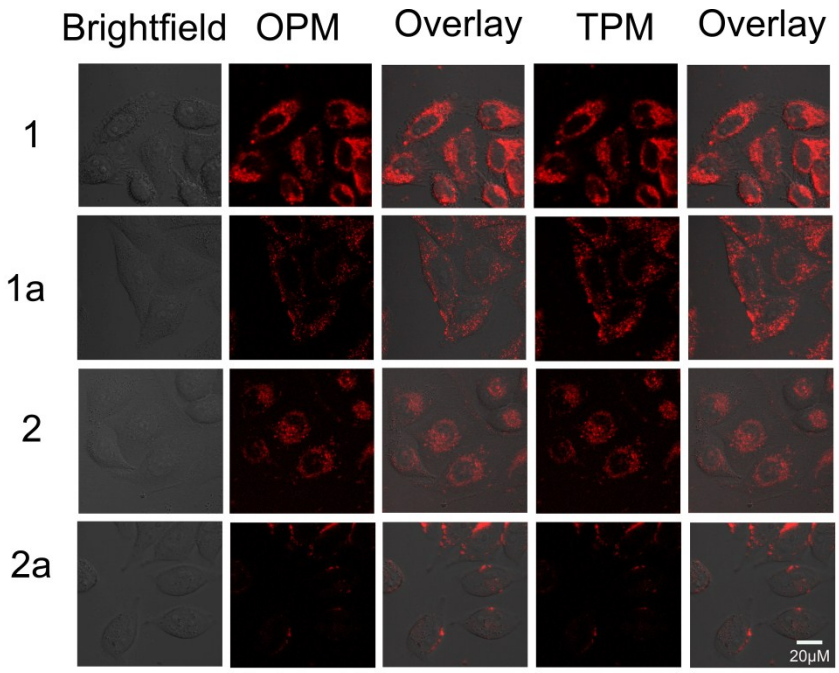

Fig. S6 One-photon (λ_{ex}= 488 nm) and two-photon (λ_{ex}= 800 nm) confocal microscopic images (λ_{em}= 620±20 nm) of Hela cells recorded after treatment with complex **1, 1a, 2, 2a** (20 μM) for 24h in the dark.

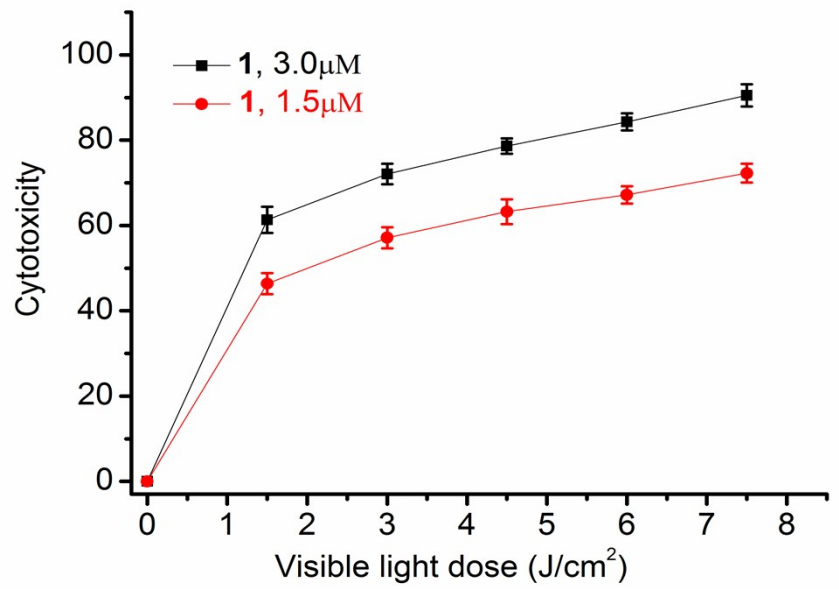

Fig. S7 Visible light (γ_{irr} = 450nm) dose-dependent cytotoxicity curve of complex **1** (3.0 μM and 1.5 μM) on monolayer Hela cells after 24h incubation in the dark. The MTT assay was performed 48h after PDT treatment. The results were expressed as the mean ±S.D. of 3 replicate trials.

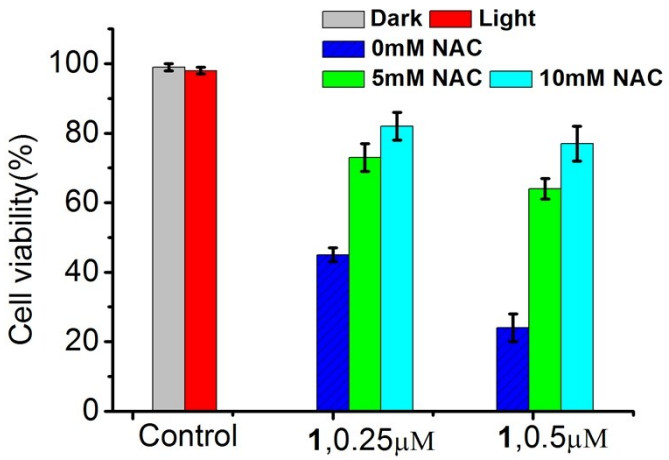

Fig.S8 Concentration-dependent inhibition of NaN_3 (5 and 10 mM) on **1**-induced Hela cell death during PDT (irradiated at 450 nm 25mW·cm^{-2}, 5min).

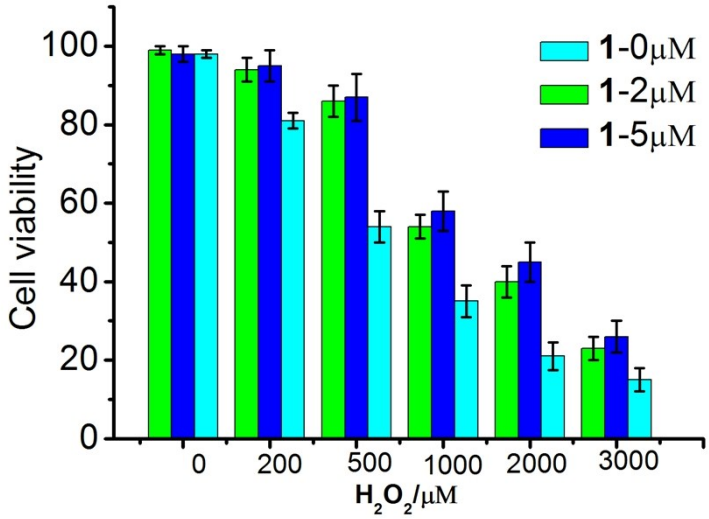

Fig. S9 Cell viability evaluation of Hela cell co-inbubated with H_2O_2 for 30 min at the indicated concentration, the cell were incubated with complex **1** (2 and 5μM) for 24h before addition of H_2O_2.

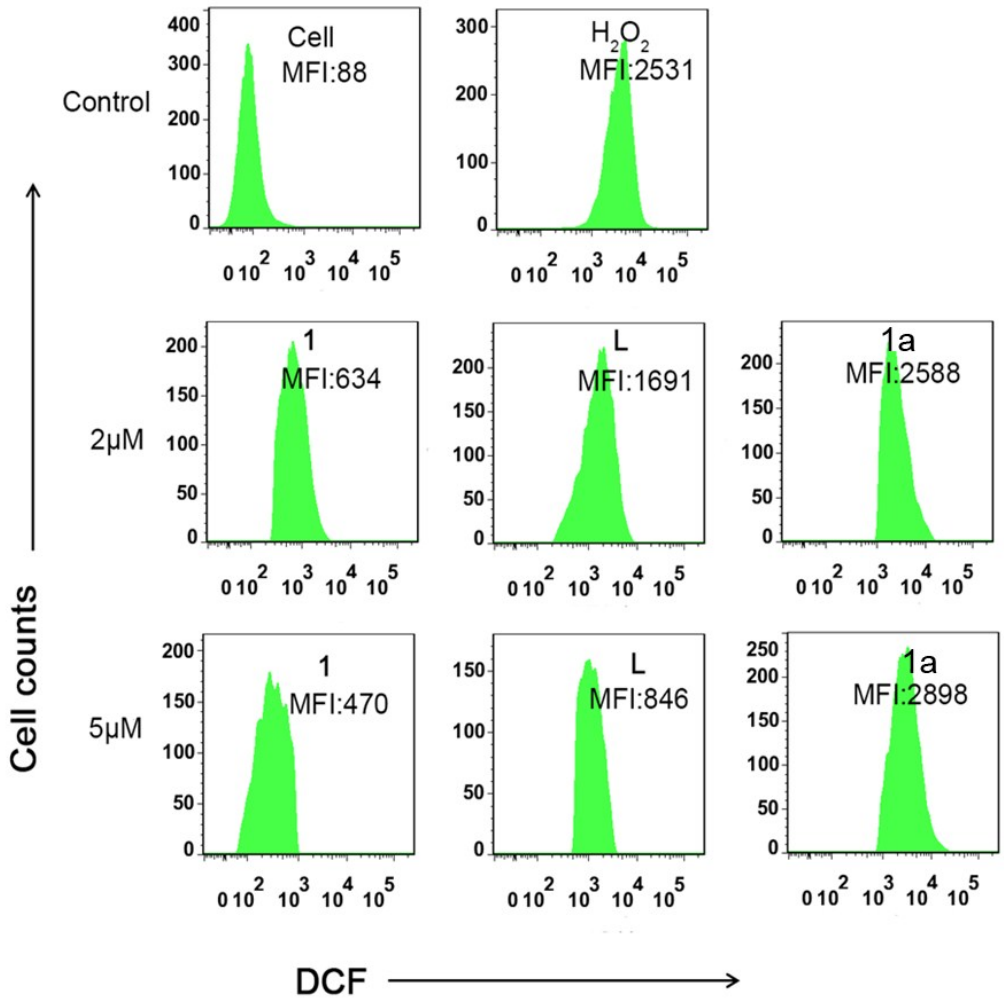

Fig.S10 Analysis of ROS levels by flow cytometry after HeLa cells were treated complexes **1**,**1a** and **L** at the indicated concentrations for 24 h and then simulated with H_2O_2 (2 mM, 30 min).

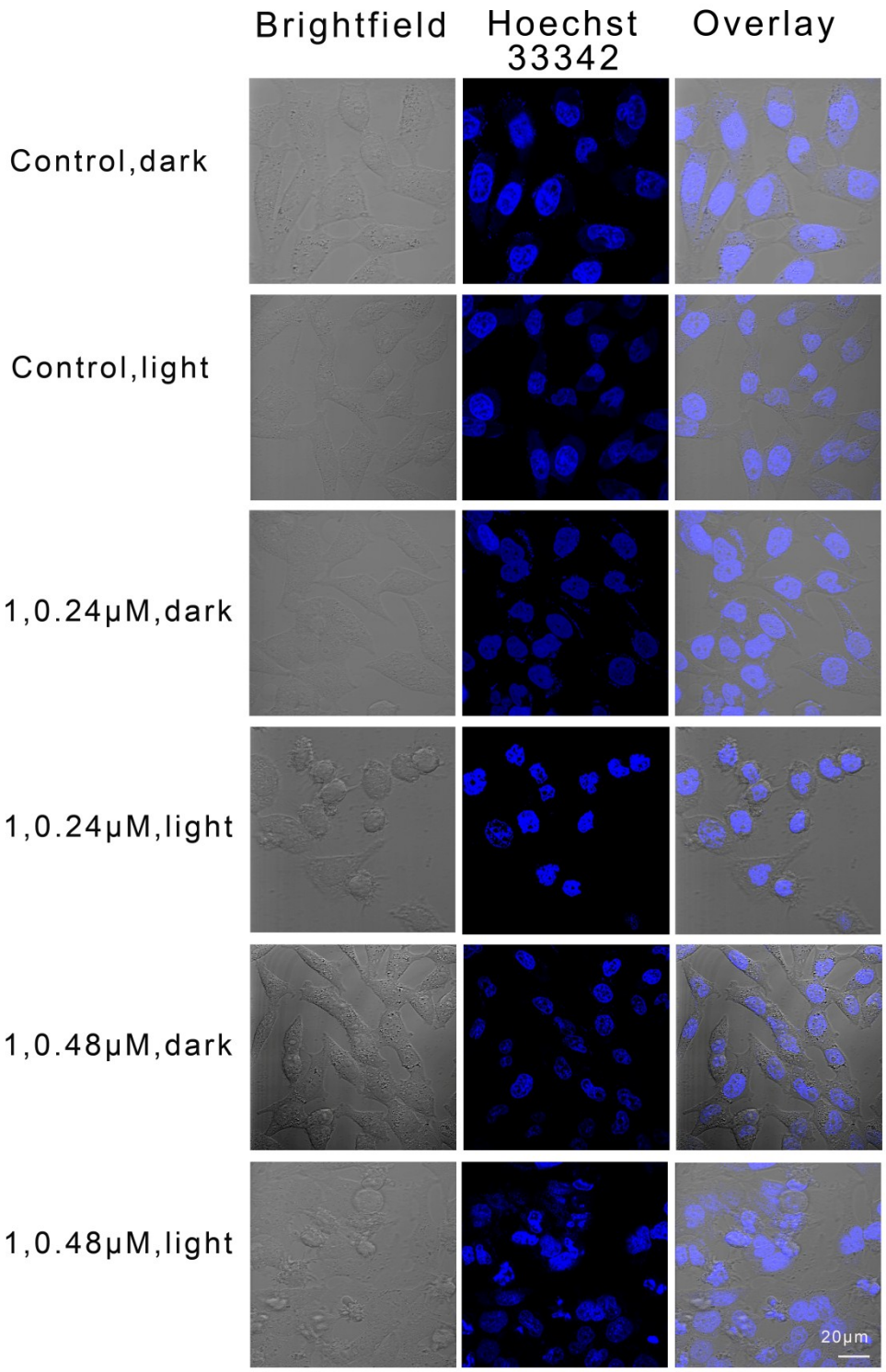

Fig. S11 Characterization of apoptosis induced by complex **1** using Hoechst 33342 staining. **Dark:** Cells were incubated with [1]complex **1** for 24 h. **Light**: Cells were incubated [2]with complex **1** for 24 h in the dark, then irradiated at 450 nm (25mW·cm^{-2}, 5 min), and visualized immediately by confocal microscopy.

Table S1 Crystallographic data of Ligand L

Compound	**L**
Empirical formula	$C_{22}H_{25}N_4O_2$
Molecular weight	378
Description	Block,orange
Temperature (K)	293 (2)
Crystal size (mm)	0.2×0.2×0.2
Λ (Å)	0.71073
Crystal system	Monoclinic
Space group	P21/c
A (Å)	7.8442 (7)
B (Å)	23.492 (2)
C (Å)	12.4040 (11)
A (°)	90
B (°)	120.785 (5)
Γ (°)	90
Volume, Å^3	1964.2 (3)
Z	4
Absorption coefficient(mm^{-1})	0.084
F(000)	804.0
Θ range (deg)	2.77-27.480
Completeness to θ_{max}	0.985
Density(calcd)(mg/m^{-3})	1.273
[R_{int}]	0.0487
Reflections collected/unique	14222/4434
R1[a][I > 2σ(I)]	0.0646
wR2[a]	0.2271
GOF[b]	1.066

[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 L

	Ligand	
bond lengths (Å)	N1 – C1	1.328 (3)
	N2 – C11	1.312 (4)
	O2 – N4	1.288 (2)
	O1 – C13	1.238 (3)
	N3 –C14	1.468 (3)
	N3 – C13	1.340 (3)
Bond angles (deg)	C14 – N3– C13	120.48 (19)
	O2 – N4 – C17	116.19 (18)
	O1 –C13 –C7	121.1 (2)
	N1 – C1	1.328 (3)

Table S3 Photophysical data of complexes 1-2, 1a-2a[a]

Compounds	Medium	$\lambda_{abs,\ max}$	$\lambda_{em,\ max}$	Φ_{em}[b]	τav[c]/ns
1	PBS	457	623	0.082	739.07
	CH_3CN	456	617	0.070	174.65
1a	PBS	435	628	0.068	715.36
	CH_3CN	432	625	0.052	174.19
2	PBS	450	622	0.066	529.32
	CH_3CN	449	615	0.048	144.68
2a	PBS	450	627	0.064	527.18
	CH_3CN	449	614	0.054	139.21
$[Ru(bpy)_3](PF6)_2$	PBS	-	632	0.042	363.99
	CH_3CN	-	615	0.062	161.68

[a] Quantum yields of luminescence at room temperature were calculated according to literature procedures.[3]All emission decays were obtained on freshly prepared samples at 2×10^{-5} M concentration. [b] Solutions of $[Ru(bpy)_3](PF_6)_2$ were used as the standard, PBS (Φ_m = 0.042)[4], CH_3CN (Φ_m =0.062)[5] and CH_2Cl_2 (Φ_m = 0.059)[6]. [c] Decay curves of compounds were recorded by an Edinburgh FLS 920 spectrometer.

Table S4 The $^1O_2$ quantum yields (Φ_Δ) of 1, 1a, 2, 2a in aerated DMSO

Compounds	Φ_Δ[a]
	450 nm
1	0.80
1a	0.71
2	0.58
2a	0.47

[a] MB was used as the reference in the Φ_Δ measurements (0.52 in aerated DMSO).

Table S5 Cellular uptake efficiency of 1,1a,2,2a measured by ICP-MS.

Complex	Amount of iridium (ng/10^6 cells)	
	12h	24h
1	65.13±0.79	147.97±0.25
1a	16.25±0.85	65.92±0.83
2	1.61±0.12	2.78±0.25
2a	1.57±0.17	3.95±0.14

References:

[1] J. M. Wessels, C. S. Foote, W. E. Ford and M. A. Rodgers, *Photochem. Photobio.*,2008, **65**, 96-102.
[2] T. Mosmann, *J. Immunol. Methods.*, 1983, **65**, 55-63.
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