

Supporting Information

**Tracking mitochondrial pH fluctuation during cell apoptosis with  
two-photon phosphorescent iridium(III) complexes**

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## Experimental Section

### Materials

The reagents and buffer components were purchased from commercial sources. IrCl<sub>3</sub>, 2-phenylquinoline (2pq), *N*-bromosuccinimide, 2,2'-azobis(2-methylpropionitrile), morpholine, CCl<sub>4</sub>, 5,5'-dimethyl-2,2'-bipyridine, cyanide *m*-chlorophenylhydrazone (CCCP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 2-ethoxyethanol were purchased from Sigma and used as received. The mitochondrial imaging agent MitoTracker<sup>®</sup> Red FM (MTR) and lysosomal imaging agent LysoTracker<sup>®</sup> Red DND-99 (LTR) were purchased from Invitrogen. 2-Phenylbenzo[*d*]thiazole (pbt), [Ir(2pbt)<sub>2</sub>Cl]<sub>2</sub> and [Ir(2pq)<sub>2</sub>Cl]<sub>2</sub> were obtained by following the reported methods.<sup>1,2</sup> DMSO was used to dissolve the complexes and the final DMSO concentration was less than 1% (v/v) in the biological experiments. Britton-Robison (B-R) buffer was prepared from a mixture of CH<sub>3</sub>COOH (0.04 M), H<sub>3</sub>PO<sub>4</sub> (0.04 M), and H<sub>3</sub>BO<sub>3</sub> (0.04 M) and was titrated to the desired pH with HCl (0.2 M) or NaOH (0.2 M).<sup>3</sup> The high K<sup>+</sup> buffer consisted of a mixture of 10.0 μM nigericin sodium, 20 mM NaOAc, 20 mM HEPES, 5 mM glucose, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 120 mM KCl, and 30 mM NaCl, and the mixture was titrated to the desired pH with HCl or NaOH.<sup>4</sup>

### General instruments

<sup>1</sup>H NMR spectra were performed on a nuclear magnetic resonance spectrometer (Mercury-Plus 300, Varian, USA). Electrospray ionization mass spectra (ESI-MS) were obtained by an LCQ system (Finnigan MAT, USA). Microanalyses (C, H and N) were determined by a Perkin-Elmer 240Q elemental analyzer. Tetramethylsilane (TMS) was used as reference for the chemical shifts in the <sup>1</sup>H NMR spectra. The time-resolved emission measurements, emission spectra and UV-Vis spectra were obtained with an FLS 920 combined fluorescence-lifetime and steady-state spectrometer, a spectrofluorophotometer (Perkin-Elmer LS 55) and a spectrophotometer (Perkin-Elmer Lambda 850) at 25 °C, respectively. The TPA cross sections were obtained by an Opolette<sup>™</sup> 355II instrument (pulse width ≤ 100 fs, 80 MHz repetition rate, tuning range 710-850 nm, Spectra Physics

Inc., USA).<sup>5,6</sup> The *n*-octanol/water partition coefficients for **P1** and **P2** were determined by ultraviolet spectrophotometry with the shake-flask method.<sup>2</sup> The luminescent quantum yield of the standard [Ru(bpy)<sub>3</sub>]<sup>2+</sup> was 0.028 in an aerated aqueous solution.<sup>7</sup> Origin 8 was used to process all data.

## Synthesis of 5,5'-bis(morpholinomethyl)-2,2'-bipyridine (**L**)

A mixture of *N*-bromosuccinimide (4.50 g, 25 mmol), 5,5'-dimethyl-2,2'-bipyridine (1.84 g, 10 mmol), 2,2'-azobis(2-methylpropionitrile) (0.33 g, 2 mmol) and CCl<sub>4</sub> (50 mL) was refluxed under argon for 3 h. The warm reaction mixture was filtered to remove the residues, and the solvent was evaporated.<sup>8</sup> Without purification, the solid was next mixed with potassium carbonate (4.14 g, 30 mmol) and morpholine (2 mL) in CH<sub>3</sub>CN (50 mL) and then refluxed under argon for 6 h. After the reaction was cooled to R.T. and filtered, the filtrate was evaporated under reduced pressure. Finally, the crude product was purified by column chromatography (dichloromethane/ethyl acetate) to obtain **L** (2.69 g, yield = 76%). Anal. Calcd. for C<sub>20</sub>H<sub>26</sub>N<sub>4</sub>O<sub>2</sub> (%): C, 67.77; H, 7.39; N, 15.81. Found: C, 67.46; H, 7.43; N, 15.71. <sup>1</sup>H NMR (300 MHz, d<sub>6</sub>-DMSO) δ 8.56 (s, 2H), 8.32 (d, *J* = 8.7 Hz, 2H), 7.84 (d, *J* = 8.7 Hz, 2H), 3.56 (s, 12H), 2.38 (s, 8H). ESI-MS: *m/z* = 355.2 [M+H]<sup>+</sup>, 377.1 [M+Na]<sup>+</sup>.

## Synthesis of the iridium(III) complexes

**P1** and **P2** were prepared using a modified method from the literature.<sup>9</sup> Briefly, [Ir(2pq)<sub>2</sub>Cl]<sub>2</sub> (or [Ir(pbt)<sub>2</sub>Cl]<sub>2</sub>, 0.1 mmol), the ligand (**L**, 0.2 mmol), 20 mL of DCM and methanol (1:1, v/v) were mixed in a 50 mL round-bottom flask and heated at 65 °C under argon overnight. The reaction was cooled to R.T., the solvent was evaporated and column chromatography was performed over alumina to purify the crude product with DCM/ethanol as the eluent. Yield: 65-75%. ESI-MS, <sup>1</sup>H NMR and elemental analysis were used to characterize the complexes.

[Ir(pbt)<sub>2</sub>L]Cl (**P1**): Anal. Calcd. for C<sub>46</sub>H<sub>42</sub>ClIrN<sub>6</sub>O<sub>2</sub>S<sub>2</sub> (%): C, 55.10; H, 4.22; N, 8.38. Found: C, 54.83; H, 4.24; N, 8.34. <sup>1</sup>H NMR (300 MHz, d<sub>6</sub>-DMSO) δ 8.76 (d, *J* = 8.3 Hz, 2H), 8.25 (d, *J* = 7.6 Hz, 2H), 8.15 (d, *J* = 8.4 Hz, 2H), 8.11-8.03 (m, 4H), 7.43 (t, *J* = 8.1

Hz, 2H), 7.20-7.13 (m, 4H), 6.96 (t,  $J = 7.5$  Hz, 2H), 6.34 (d,  $J = 7.5$  Hz, 2H), 6.17 (d,  $J = 8.4$  Hz, 2H), 3.69-3.41 (m, 12H), 2.14 (t,  $J = 8.2$  Hz, 8H). ESI-MS:  $m/z = 967.4$  [M-Cl]<sup>+</sup>.

**[Ir(2pq)<sub>2</sub>L]Cl (P2)**: Anal. Calcd. for C<sub>50</sub>H<sub>46</sub>ClIrN<sub>6</sub>O<sub>2</sub> (%): C, 60.62; H, 4.68; N, 8.48. Found: C, 60.32; H, 4.71; N, 8.44. <sup>1</sup>H NMR (300 MHz, d<sub>6</sub>-DMSO)  $\delta$  8.58 (dd,  $J = 20.0, 8.9$  Hz, 4H), 8.38 (dd,  $J = 19.3, 8.0$  Hz, 4H), 8.10 (s, 2H), 7.95 (t,  $J = 8.8$  Hz, 4H), 7.43 (t,  $J = 7.5$  Hz, 2H), 7.26-7.15 (m, 4H), 7.08 (ddd,  $J = 8.7, 6.9, 1.4$  Hz, 2H), 6.84 (t,  $J = 7.4$  Hz, 2H), 6.41 (d,  $J = 7.0$  Hz, 2H), 3.61-3.41 (m, 12H), 2.10 (t,  $J = 4.3$  Hz, 8H). ESI-MS:  $m/z = 955.5$  [M-Cl]<sup>+</sup>.

## Computational modelling

All calculations were carried out by employing the Gaussian 09 program package.<sup>10</sup> The geometries were optimized with the semi-empirical method PM6,<sup>11</sup> and the single-point energies were further refined at the level of B3LYP/6-311++G\*\* (SDD basis sets with Stuttgart/Dresden ECP for the Ir atoms) the gas phase.<sup>12,13</sup> In addition, an empirical Grimme's dispersion correction with Becke-Johnson damping (GD3BJ) was considered in the single-point calculations.<sup>14</sup> Kohn-Sham frontier orbitals energies and locations were analyzed to rationalize the experimental observations.

## Cell culture conditions

HeLa cells were cultured in a mixed medium of 90% DMEM and 10% FBS under conditions of 37 °C and 5% CO<sub>2</sub>.

## Cytotoxicity

Seeded into 96-well plates for 24 h, HeLa cells (1×10<sup>4</sup> cells/well) were incubated with 5  $\mu$ M of complexes with increasing incubation times. MTT (20  $\mu$ L, 5 mg/mL) was used to stain the viable cells in the plates for 4 h. Removed the media, DMSO (200  $\mu$ L/well) was added and the optical density was measured at 595 nm by the microplate reader (Tecan Infinite M200). The wells containing cells incubated without complexes were set as 100% cell survival.

## **Imaging of HeLa cells**

Glass bottom dishes (35 mm, Corning) were used to plate HeLa cells for 24 h. For mitochondrial co-localization, after incubation with 5  $\mu\text{M}$  of Ir(III) complexes for 30 min, MTR was added and further incubated for 30 min. Media were replaced and cell imaging was performed. For lysosome colocalization, after treatment with the Ir(III) complexes for 30 min, LTR was treated, and cells were incubated for another 30 min. The media were replaced, and cell imaging was again performed. For the intracellular pH calibration, the cells were treated with pH sensors for 30 min, washed 3 times with PBS, and treated with high  $\text{K}^+$  buffer at various pH values. Cell imaging was then performed after 30 min. For the observation of pH before and after apoptosis, after incubation with Ir(III) complexes for 30 min, and washed 3 times with PBS, the cells were imaged, treated with CCCP (20  $\mu\text{M}$ ) for 10 min, and imaged again. Confocal microscope (Zeiss LSM 710 NLO, 63 $\times$ /NA 1.4 oil immersion objective) was used to image the cells. The excitation wavelengths were 405 nm (for **P1** and **P2**), 730 nm (for two-photon imaging) and 543 nm (for MTR and LTR). The collected emission signal was  $550 \pm 20$  nm (for **P1** and **P2**) or  $620 \pm 20$  nm (for MTR and LTR).

## **Cellular uptake**

In the uptake experiments, HeLa cells in the exponentially growing phase were harvested to make a single-cell suspension, with which a 10 cm tissue culture plate was covered and cultured at 37 °C. After 24 h, the HeLa cells were treated with iridium(III) complexes (5  $\mu\text{M}$ ) for 30 min, the HeLa cells were divided into two portions. One portion was subjected an extraction process with a mitochondrial extraction kit of Thermo. All samples were digested with  $\text{HNO}_3$  (60%) for more than 24 h, and diluted to a final volume of 10 mL with Milli-Q water. An inductively coupled plasma mass spectrometer was used to determine the content of iridium.

## **Cellular uptake mechanism**

After pretreatment with inhibitors and **P1** and **P2**, the cells were imaged by laser confocal scanning microscopy.<sup>15</sup> The excitation wavelength for **P1** and **P2** was 730 nm, and an emission filter of  $550 \pm 20$  nm was used. For normalized incubation, HeLa cells were treated with 5  $\mu$ M complexes at 37 °C for 30 min. For low temperature inhibition, the cells were incubated with at 4 °C complexes (5  $\mu$ M) for 30 min. For metabolic inhibition, the cells were pretreated at 37 °C in PBS with 5  $\mu$ M oligomycin and 50 mM 2-deoxy-D-glucose for 1 h and treated at 37 °C with 5  $\mu$ M of complexes for 30 min. For endocytic inhibition, the endocytic inhibitor NH<sub>4</sub>Cl (50 mM) or chloroquine (50  $\mu$ M) was used to pretreat the cells for 30 min, which were subsequently treated with 5  $\mu$ M of the complexes for an additional 30 min.

## Imaging of multicellular tumor spheroid

A 3D multicellular tumor spheroid (MCTS) was cultured for imaging by the liquid overlay method.<sup>16</sup> For achieve single-cell suspensions, exponentially growing HeLa cells were digested by trypsin. In 96-well plates previously covered in DMEM with agarose (1.0%, 50  $\mu$ L), approximately 6000 suspended cells were seeded. The MCTSs were harvested after 72 h of incubation at 37 °C with 5% CO<sub>2</sub>. To investigate the depth of the complexes under one- and two-photon lasers, the 3D MCTSs with diameters of 450-550  $\mu$ m were incubated with **P1** and **P2** (5  $\mu$ M) for 6 h. The MCTSs were imaged by a confocal microscope (Zeiss LSM 710 NLO, 10 $\times$  objective). The excitation wavelengths were 405 nm (for **P1** and **P2**) and 730 nm (for two-photon imaging). An emission filter of  $550 \pm 20$  nm was used.

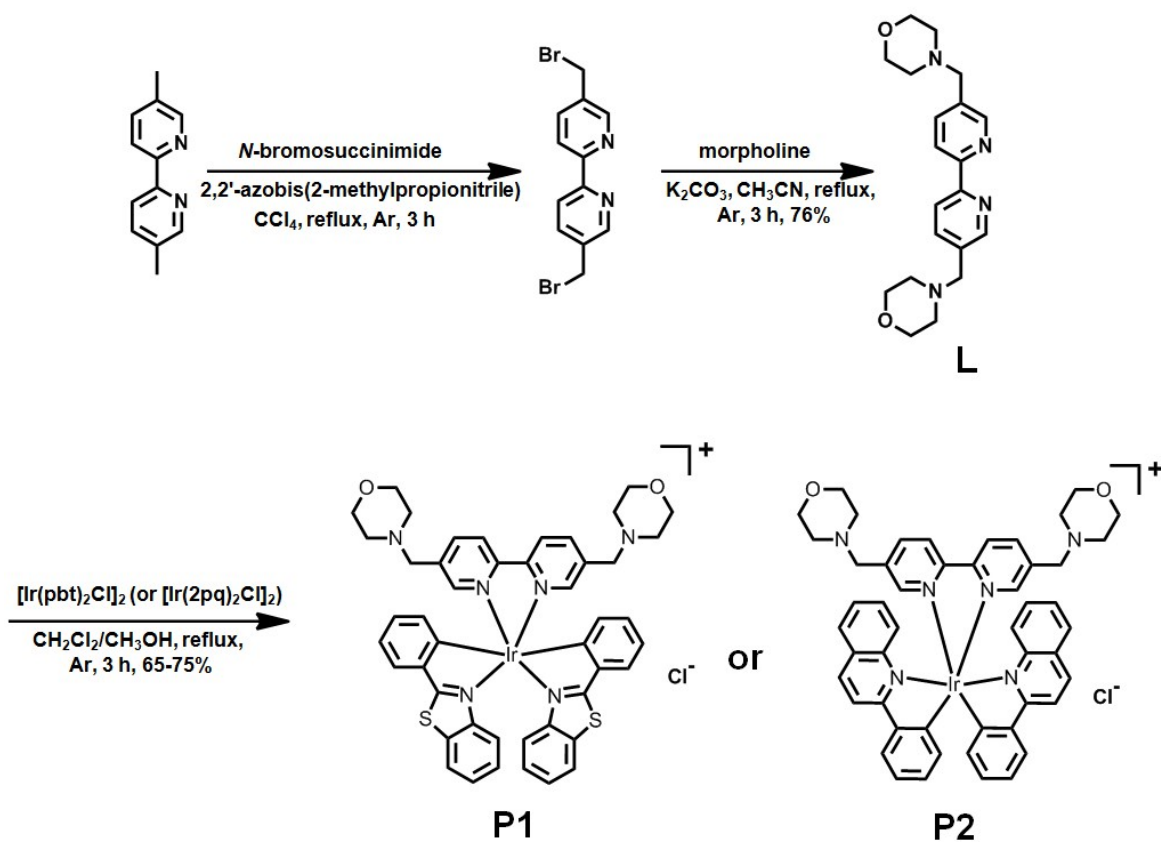
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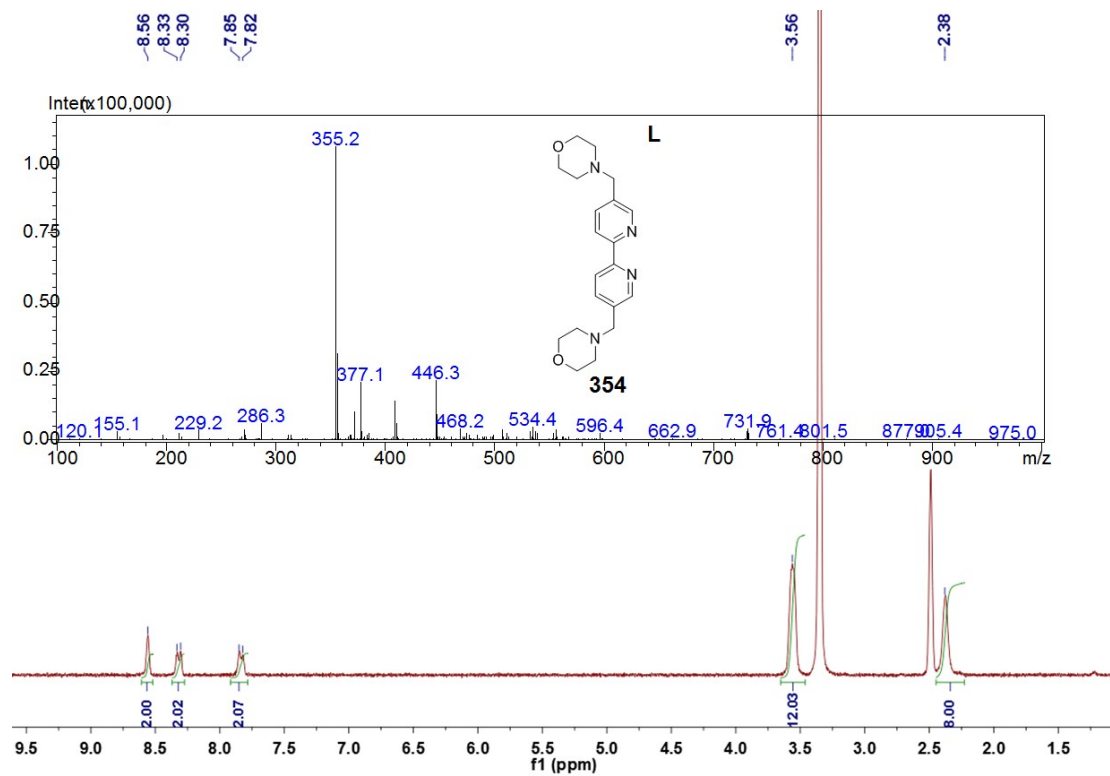
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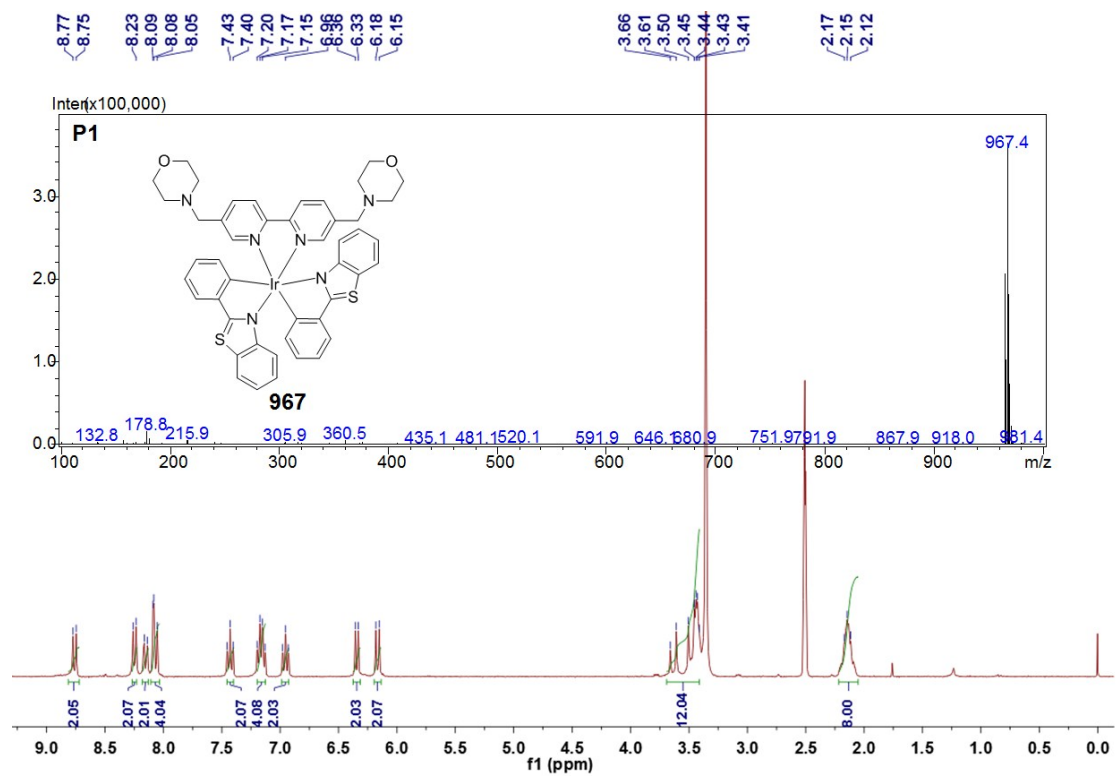
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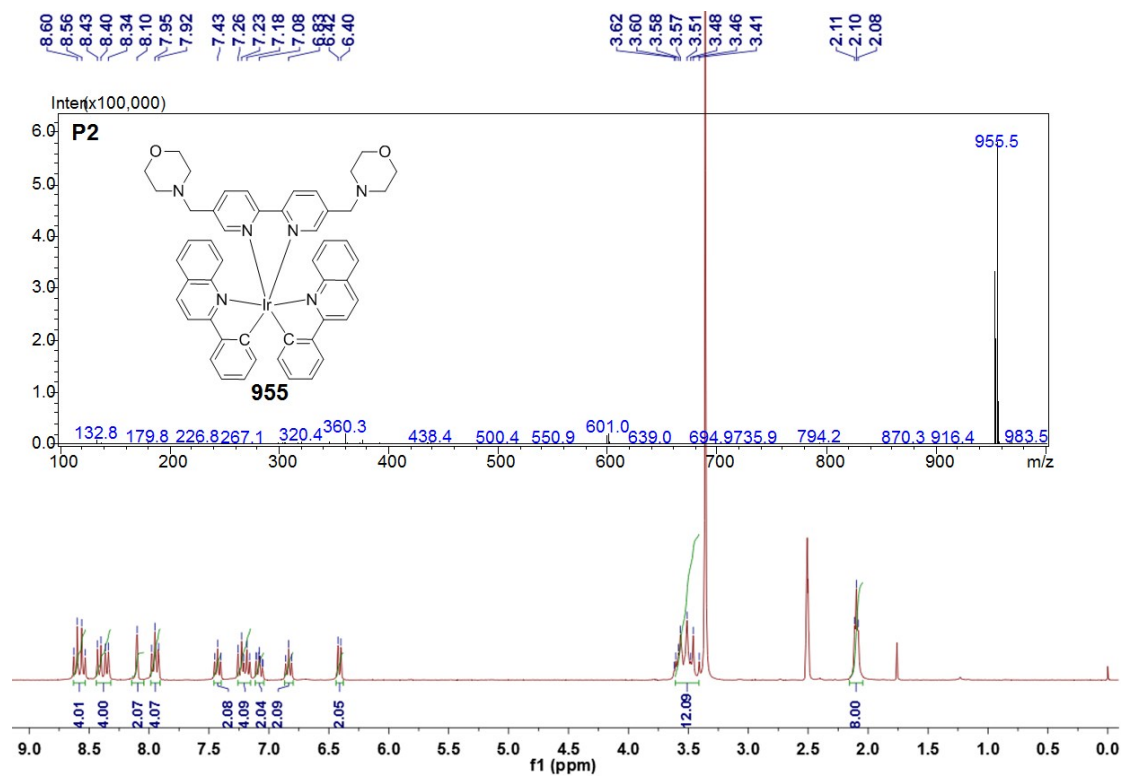
**Scheme S1.** Synthetic routes to **P1** and **P2**.



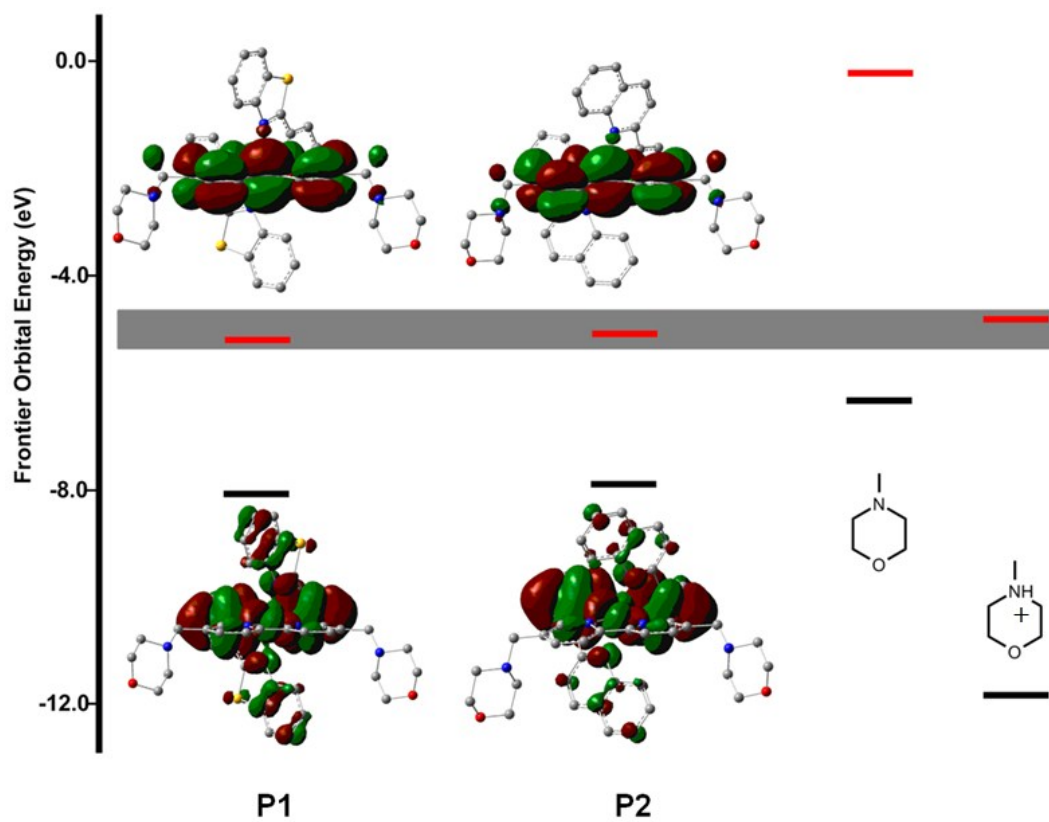
**Fig. S1** ESI-MS spectrum and <sup>1</sup>H NMR spectrum of ligand **L**.



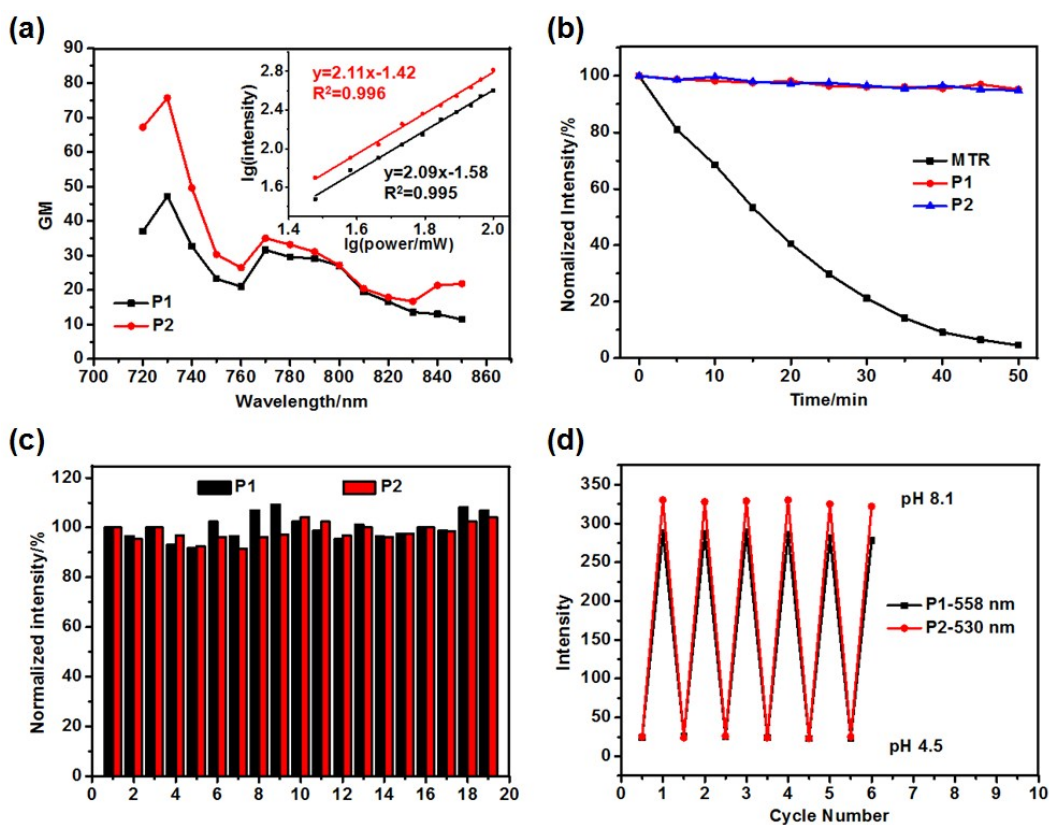
**Fig. S2** ESI-MS spectrum and <sup>1</sup>H NMR spectrum of **P1**.



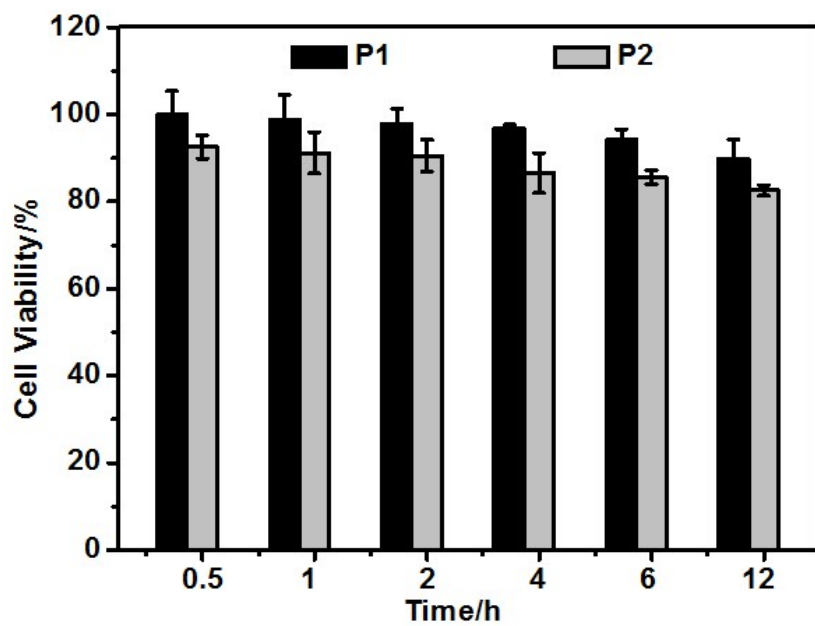
**Fig. S3** ESI-MS spectrum and  $^1\text{H}$  NMR spectrum of **P2**.



**Fig. S4** Comparison of the calculated HOMO and LUMO energies of **P1**, **P2**, a model morpholine moiety, and the protonated morpholine moiety, as well as the electron density distribution in the HOMOs and LUMOs of **P1** and **P2**.

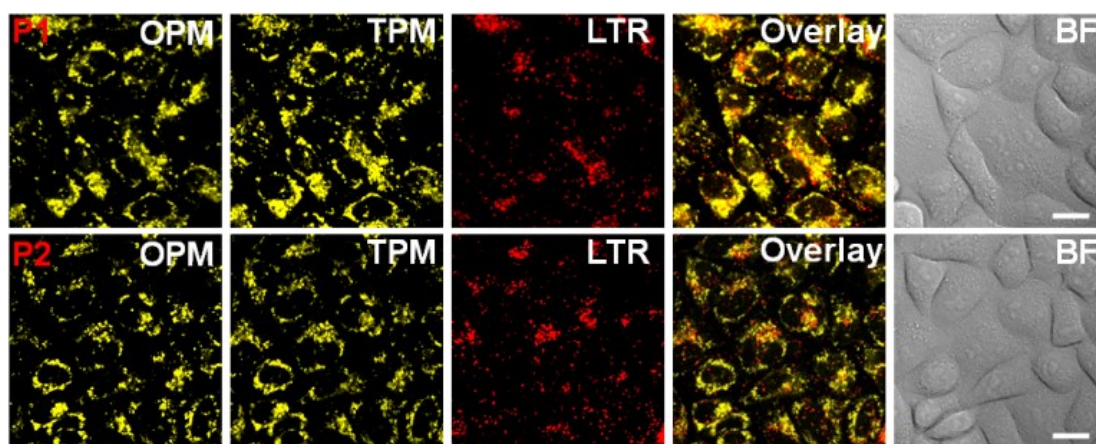


**Fig. S5** (a) Two-photon absorption cross-sections of **P1** and **P2**. (b) Florescence (or phosphorescence) intensity decay of MTR, **P1** and **P2** in buffer during irradiation by a 300 W iodine-tungsten lamp. (c) Emission intensities of the complexes in the presence of 200  $\mu$ M cations, anions and 1.0 mM GSH and Cys in a buffer solution. 1. complexes, 2.  $\text{Li}^+$ , 3.  $\text{Na}^+$ , 4.  $\text{K}^+$ , 5.  $\text{Mg}^{2+}$ , 6.  $\text{Ca}^{2+}$ , 7.  $\text{Fe}^{3+}$ , 8.  $\text{Co}^{2+}$ , 9.  $\text{Cu}^{2+}$ , 10.  $\text{Zn}^{2+}$ , 11.  $\text{Cl}^-$ , 12.  $\text{Br}^-$ , 13.  $\text{CO}_3^{2-}$ , 14.  $\text{NO}_3^-$ , 15.  $\text{OAc}^-$ , 16.  $\text{PO}_4^{3-}$ , 17.  $\text{SO}_4^{2-}$ , 18. GSH, 19. Cys. (d) pH reversibility study of the phosphorescence of the complexes between pH 4.5 and 8.1 in buffer solution.

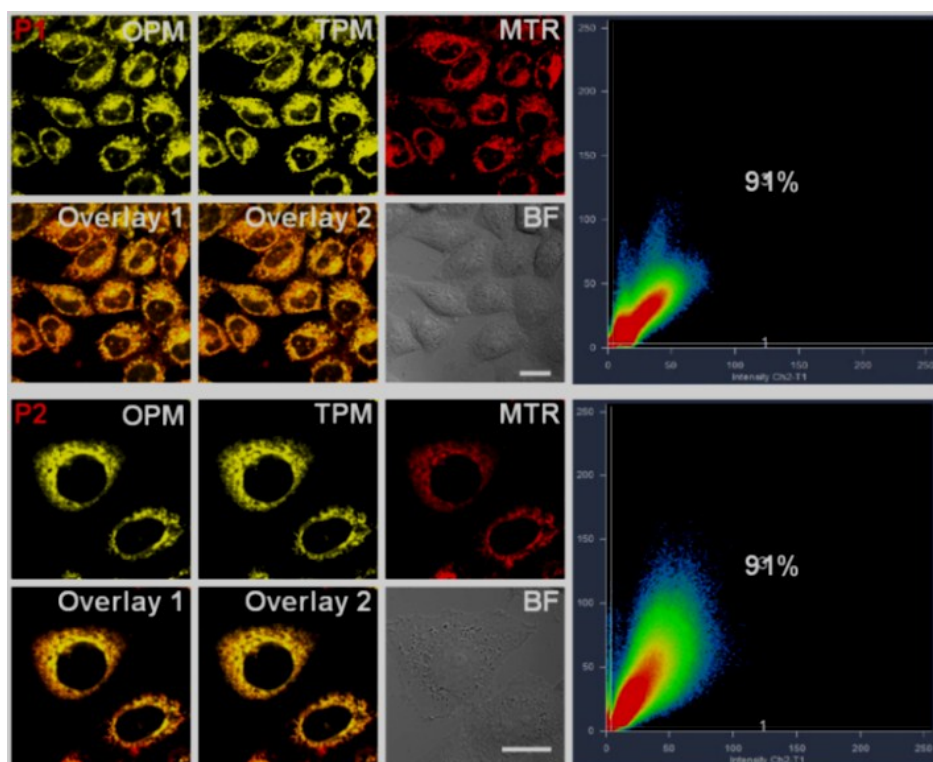


**Fig. S6** Viability of HeLa cells incubated with 5  $\mu$ M **P1** and **P2** for different length of time.

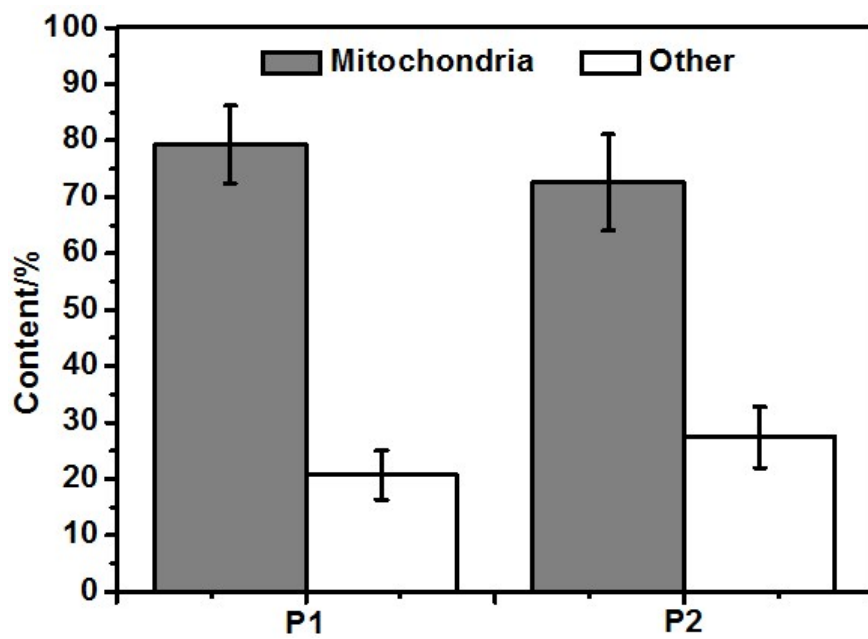




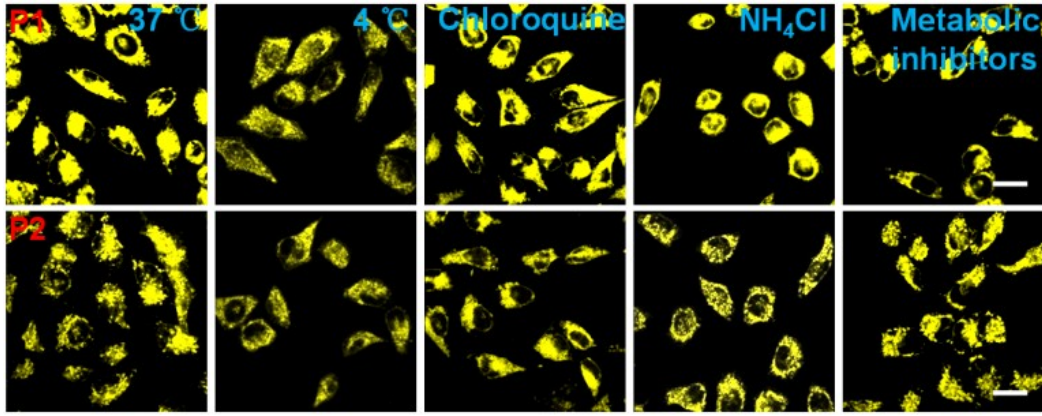
**Fig. S7** One-photon microscopy (OPM) and two-photon microscopy (TPM) images of HeLa cells co-labeled with the complexes ( $5 \mu\text{M}$ , 0.5 h,  $\lambda_{\text{OP-ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{TP-ex}} = 730 \text{ nm}$ ,  $\lambda_{\text{em}} = 550 \pm 20 \text{ nm}$ ) and LTR ( $50 \text{ nM}$ , 0.5 h,  $\lambda_{\text{ex}} = 543 \text{ nm}$ ,  $\lambda_{\text{em}} = 620 \pm 20 \text{ nm}$ ). Overlay: Overlay of TPM and LTR. BF: bright-field images. Scale bar:  $20 \mu\text{m}$ .



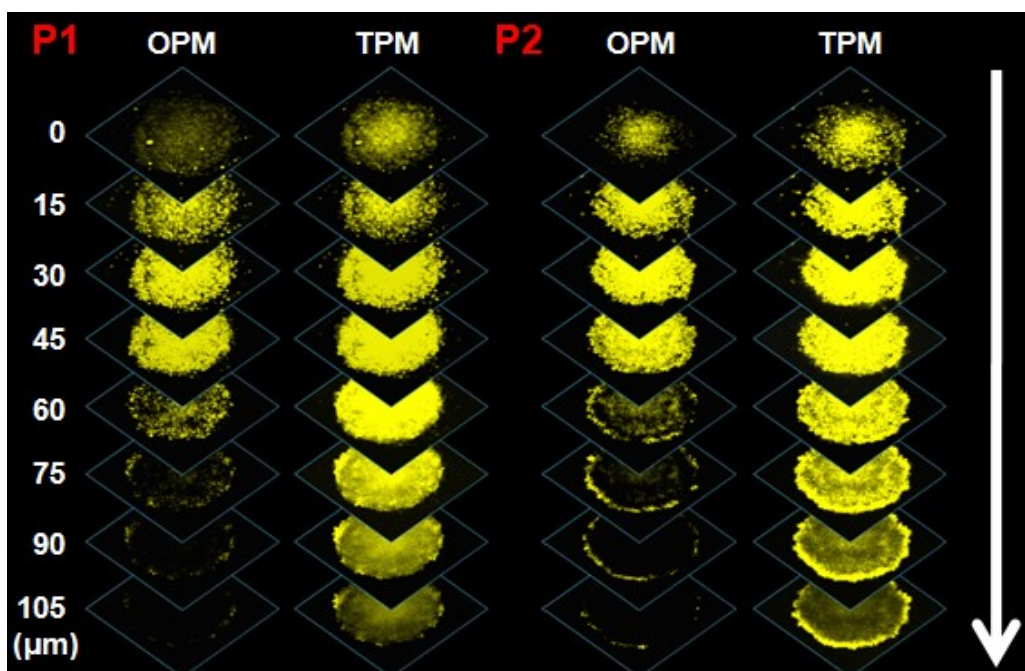
**Fig. S8** One-photon microscopy (OPM) and two-photon microscopy (TPM) images of HeLa cells co-labeled with complexes ( $5 \mu\text{M}$ ,  $0.5 \text{ h}$ ,  $\lambda_{\text{OP-ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{TP-ex}} = 730 \text{ nm}$ ,  $\lambda_{\text{em}} = 550 \pm 20 \text{ nm}$ ) and MTR ( $50 \text{ nM}$ ,  $0.5 \text{ h}$ ,  $\lambda_{\text{ex}} = 543 \text{ nm}$ ,  $\lambda_{\text{em}} = 620 \pm 20 \text{ nm}$ ). Overlay 1: Overlay of OPM and MTR. Overlay 2: Overlay of TPM and MTR. BF: bright-field images. Scale bar:  $20 \mu\text{m}$ .



**Fig. S9** Distributions of complexes **P1** and **P2** in cells.



**Fig. S10** TPM images of living HeLa cells incubated with **P1** and **P2** ( $5 \mu\text{M}$ ,  $0.5 \text{ h}$ ,  $\lambda_{\text{ex}} = 730 \text{ nm}$ ,  $\lambda_{\text{em}} = 550 \pm 20 \text{ nm}$ ) under different conditions. Scale bar:  $20 \mu\text{m}$ .



**Fig. S11** One- and two-photon phosphorescent images of 3D tumor spheroids after incubation with **P1** and **P2** (5  $\mu\text{M}$ , 6 h,  $\lambda_{\text{OP-ex}}$  = 405 nm,  $\lambda_{\text{TP-ex}}$  = 730 nm,  $\lambda_{\text{em}}$  =  $550 \pm 20$  nm).

**Table S1** Photophysical data for **P1** and **P2** at 298 K.

Complexes	$\lambda_{ab}$ <sup>a</sup>	$\epsilon$ <sup>b</sup>	$\lambda_{em}$ <sup>c</sup>	$\phi$ <sup>d</sup>	$\tau/ns$ <sup>e</sup>	$\delta/GM$ <sup>f</sup>
<b>P1</b>	406	1.56	530,567	0.097	175	47.2
<b>P2</b>	426	1.12	558	0.145	154	75.7

<sup>a</sup> $\lambda_{ab}$  maximum values of the one-photon absorption in nm. <sup>b</sup> Extinction coefficient in  $1 \times 10^4 M^{-1} \cdot cm^{-1}$ . <sup>c</sup> $\lambda_{em}$  maximum values of the one-photon emission spectra in nm. <sup>d</sup> Phosphorescent quantum yield. <sup>e</sup> Life time. <sup>f</sup> Two-photon absorption cross section at 730 nm, measured in methanol.