Supporting information

Halides Tuning Subcellular-Targeting in Two-Photon Emissive Complexes via Different Uptake Mechanisms

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Experimental section

Measurements and apparatus

All chemical agents were obtained from Aladdin, then dried and purified by standard methods. Commercial bio-dyes used for organelles staining were bought from Sigma-Aldrich. IR spectra were recorded on a NEXUS 870 (Nicolet) spectrophotometer in the 4000-400 cm^{-1} region with samples prepared as KBr pellets. Elemental analyses were performed with a Perkin-Elmer 240B instrument. MALDI-TOF mass spectra were recorded using a Bruker Autoflex III Smartbeam. ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker Avance 400 MHz spectrometer (TMS as internal standard in NMR). Melting points were measured on a FP62 instrument.

Optical measurements

UV spectra were recorded on a UV-265 spectrophotometer. One-photon fluorescence spectra were obtained using a HITACHI F-7000 spectrofluorimeter equipped with a 450 W Xe lamp. When the fluorescence measurements were taken, the concentration of samples was 1×10^{-5} mol/L with quartz cuvette (path length = 1 cm). In the measurements of emission spectra, the slit width was 5 nm. The exciting voltage of emission spectrum was 400 V. The fluorescence quantum yields (Φ) were determined by using fluorescein as the reference according to the literature method.[x] 2PA cross-section of all samples were obtained by the two-photon excited fluorescence (2PEF) method with femtosecond laser piles and a Ti:sapphire system (680-1080 nm, 80 MHz, 140 fs) as the light source. The concentration of the sample solution was 5.0×10^{-4} mol/L.

X-ray crystallography

The X-ray diffraction measurements were performed on a Bruker SMART CCD area detector using graphite monochromated Mo-K_α radiation (λ = 0.71069 Å) at 298(2)K. Intensity data were collected in the variable ω-scan mode. The structures were solved by direct methods and difference Fourier transformations. The non-hydrogen atoms were refined anisotropically and hydrogen atoms were introduced geometrically. Calculations were performed with SHELXTL-97 program package.

Computation details

The ground states for each molecule were calculated using the density functional theory level

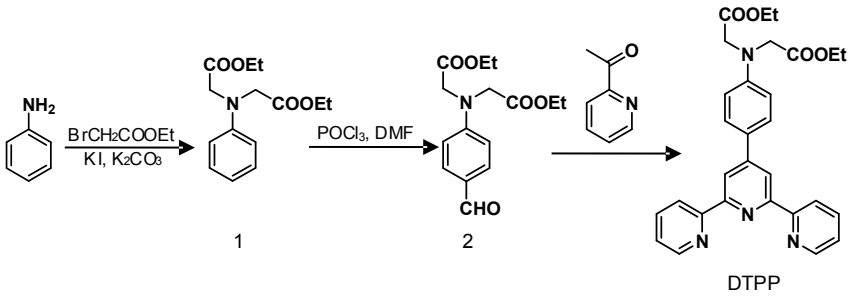
with the B3LYP functional employing a 6-31G* basis set. No symmetry or internal coordinate constraints were applied during optimization. The absorption energies were investigated by time-dependent density functional theory (TD-DFT). All calculations were performed by the use of the Gauss 03 suite of programs.

Two photon cross section

The two-photon absorption spectra of the probes are determined by two-photon induced luminescence method by using fluorescein as reference. The two-photon fluorescence measurements were performed in fluorometric quartz cuvettes. The experimental fluorescence excitation and detection conditions are conducted with negligible reabsorption processes, which can affect 2PA measurements. The two-photon absorption cross section of the probe is calculated at each wavelength according to the equation below:

$$\delta = \delta_{ref} \frac{\Phi_{ref}}{\Phi} \frac{C_{ref}}{C} \frac{n_{ref}}{n} \frac{F}{F_{ref}}$$

Here, the subscripts *ref* stands for the reference molecule. δ is the 2PA cross-section value, c is the concentration of solution, n is the refractive index of the solution, F is the integrated area of the detected two-photon-induced fluorescence signal, and Φ is the fluorescence quantum yield.



Scheme S1. Synthesis of ligand DTPP.

Synthesis of compound 1

Aniline 1.21g (13.02 mmol) and 50 mL dry acetonitrile were added to the 150 mL three-neck flask. Stirred at room-temperature and added K_2CO_3 5.01g (30 mmol) and KI 4.71g (28.38 mmol) under the N_2 atmosphere for 10 min. Subsequently, $BrCH_2COOCH_2CH_3$ 4.75g (28.38 mmol) was

dropped to the flask. The mixture was heat to 90 °C for 6 h. Tracked the reaction by TLC. After the reaction, removed the acetonitrile by distillation. The residue was dissolved in 30 dichloromethane and washed by water (2 × 20 mL). the organic solution was dried by Na_2SO_4 for 24 h. The compound was purified by silica gel chromatography column using petroleum/ethylacatate (10:1 v/v) as the eluent. 3.11g light yellow solid product **1** was collected. Yield: 90%. ^{1}H-NMR (400 MHz, d_6-DMSO): 7.19 (t, 2H), 6.75 (t, 1H), 6.60 (d, 2H), 4.17 (q, 4H), 4.11 (s, 4H), 1.23 (t, 6H).

Synthesis of compound 2

Compound **1** 3.00g (11.30 mmol) was dissolved in 4.13g (56.30 mmol) DMF and stirred for 30 min at -20 °C. After that, 8.66g (56.30 mmol) $POCl_3$ was added to the mixed solution. The mixture was heated to 60 °C for 3 h before kept at low temperature for 30 min. When the reaction was finished, the reaction solution was added to the ice-water mixture. The pH was adjusted by NaOH aqueous solution. The produce was collected by extraction using dichloromethane (2 × 40 mL). The compound was purified by silica gel chromatography column using petroleum/ethylacatate (10:1 v/v) as the eluent. 2.65g light yellow solid product **1** was collected. Yield: 80%. ^{1}H-NMR (400 MHz, d_6-DMSO): 9.71 (s, 1H), 7.70(d, 2H), 6.70 (d, 2H), 4.33 (s, 4H), 4.13 (q, 4H), 1.20 (t, 6H).

Synthesis of DTPP

Compound 2 (5.86 g, 20 mmol), 2-acetylpyridine (4.96 g, 41 mmol) and KOH (4.48 g, 80 mmol) were sequently added into 100 mL ethanol, and kept in room temperature for 15 min. Then, ammonia (25%, 124 mL) was dropped into the reaction system by three portions, and then the mixture was refluxed for 6 h. After the reaction completed, the mixture was poured into cold water, and adjusted the pH to ~5 by diluted HCl. The generated precipitate was filtered off and then dissolved into the mixture of H_2SO_4 and EtOH. After refluxing for 8 h, the mixture solution was cooled down, and then most of EtOH was removed. The residue was dispersed into 400 mL of water and adjusted pH to ~8 with K_2CO_3 and extracted three times with CH_2Cl_2. The crude product was obtained by removal of CH_2Cl_2, and the ligand DTPP was purified by silica gel chromatography column using petroleum/ethylacatate (5:1 v/v) as the eluent. Light yellow solid product **DTPP** was collected. Yield: 40%. M.p. : 163-166 ^{o}C. IR (KBr, cm^{-1}): 3053, 2983, 1753,

1607, 1523, 1466, 1392, 1262, 1178, 1020, 817, 790, 736, 520. ^{1}H-NMR (400 MHz, d_6-DMSO): δ 8.76 (d, 2H), 8.66 (m, 4H), 7.03 (t, 2H), 7.79 (d, 2H), 7.52 (t, 2H), 6.76 (d, 2H), 4.32 (s, 4H), 4.16 (q, 4H), 1.24 (t, 6H). ^{13}C-NMR (400MHz, d_6-DMSO): δ 14.1, 52.6, 60.5, 112.5, 116.6, 120.8, 124.2, 125.7, 127.6, 137.2, 148.8, 149.0, 149.2, 155.2, 155.4, 170.2. MS: 497.21 ($[M+H]^+$). Anal. Calcd. For $C_{29}H_{28}N_4O_4$: C, 70.15; H, 5.68; N, 11.28. Found: C, 70.12; H, 5.65; N, 11.30.

Synthesis of Zn(II) complexes

A solution of **DTPP** (0.19 g, 0.4 mmol) was dissolved in ethanol, then ZnX_2 (X = Cl, Br and I) (0.4 mmol) with 5 mL ethanol were added dropwise, and the reaction mixture was refluxed for 4 h. The mixture was cooled down and filtered off, and the final product was recrystallized by ethanol.

DTPP-$ZnCl_2$: Yield: 90%. M. p. 292-295 ^{o}C.

FT-IR (cm^{-1}) selected bands: 3063 (s), 2979 (s), 1733 (s), 1594 (s), 1531 (s), 1474 (s), 1203 (s), 1021 (s), 969 (s), 820 (s), 792 (s), 727 (s).

^{1}H NMR (400MHz, d_6-DMSO): δ 8.98 (m, 4H), 8.82 (s, 2H), 8.32(t, J = 7.2 Hz 2H), 8.20 (d, J = 8.5 Hz, 2H), 7.85 (s, 2H), 6.81 (d, J = 8.2 Hz, 2H), 4.38 (s, 4H), 4.18 (q, J = 7.1 Hz 4H), 1.24 (t, J = 7.1 Hz, 6H).

MALDI-TOF-MS m/z: Calculated for $([M-Cl]^+$ 595.1091. Found m/z = 595.1090.

Anal. Calcd. For $C_{29}H_{28}Cl_2N_4O_4Zn$: C, 55.04; H, 4.46; N, 8.85. Found: C, 54.86; H, 4.38; N, 8.91.

DTPP-$ZnBr_2$: Yield: 91%. M.p. > 300 ^{o}C.

FT-IR (cm^{-1}) selected bands: 3060 (s), 2980 (s), 1732 (s), 1594 (s), 1530 (s), 1475 (s), 1422 (s), 1392 (s), 1201 (s), 1020 (s), 963 (s), 819 (s), 791 (s), 726 (s).

^{1}H NMR (400MHz, d_6-DMSO): δ 8.95 (m, 6H), 8.34 (t, J = 7.1 Hz, 2H), 8.21 (d, J = 8.4 Hz, 2H), 7.89 (t, J = 6.0 Hz, 2H), 6.81 (d, J = 8.6 Hz, 2H), 4.38 (s, 4H), 4.18 (q, J = 7.0 Hz, 4H), 1.24 (t, J = 7.0 Hz, 6H).

MALDI-TOF-MS m/z: Calculated for $([M-Br]^+$ 639.0585. Found m/z = 639.0581.

Anal. Calcd. For $C_{29}H_{28}Br_2N_4O_4Zn$: C, 48.26; H, 3.91; N, 7.76. Found: C, 48.19; H, 4.02; N, 7.83.

DTPP-ZnI_2: Yield: 90%. M. p. > 300 ^{o}C.

FT-IR (cm^{-1}) selected bands: 3055 (s), 2978 (s), 1739 (s), 1596 (s), 1531 (s), 1474 (s), 1389 (s), 1208 (s), 1017 (s), 813 (s), 728 (s), 791 (s), 728 (s).

^{1}H NMR (400MHz, d_6-DMSO): δ 9.27 (s, 1H), 9.13 (d, J = 8.4, 1H), 9.02 (s, 2H), 8.90 (s, 1H), 8.40 (m, 2H), 8.26 (t, J = 7.5 Hz, 1H), 8.19 (d, J = 6.8 Hz, 1H), 7.92 (d, J = 4.3 Hz, 2H), 7.48 (t, J = 6.2 Hz, 1H), 6.90 (d, J = 8.8 Hz, 1H), 6.81 (d, J = 8.8 Hz, 1H), 4.43 (d, J = 17.6 Hz, 4H), 4.18 (m, 4H), 1.26 (t, J = 7.1 Hz, 6H).

Anal. Calcd. For $C_{29}H_{28}I_2N_4O_4Zn$: C, 42.70; H, 3.46; N, 6.87. Found: C, 42.79; H, 3.28; N, 6.88.

Cell culture

HepG2 and HELF cells were seeded on a T-25 flask, maintained in DMEM medium (Dulbecco's Modified Eagle's Medium-high glucose, D5671-SIGMA) supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum (FCS, Gibco). Cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air and sub-cultured routinely using 0.02% (w/v) EDTA trypsin (2ml, 5min 37 °C, 5% CO_2 incubation) once 100%.

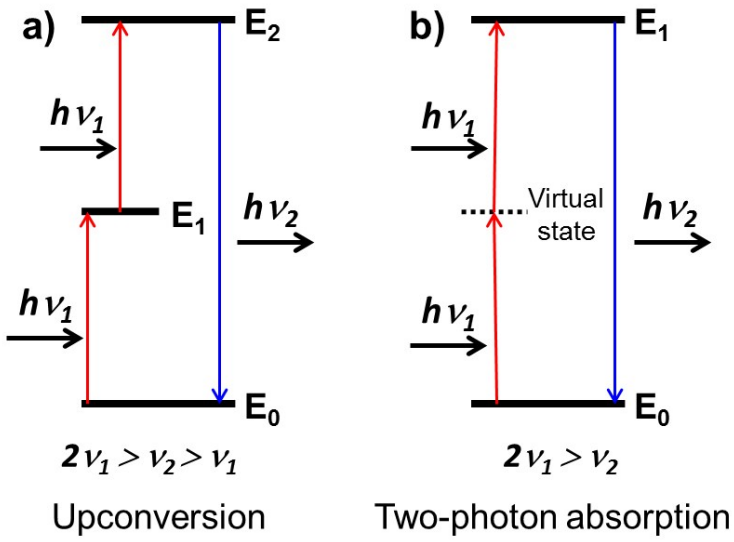
Cytotoxicity Assays in Cells

To ascertain the cytotoxic effect of all the compounds treatment over a 24 h period, the 5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay was performed. HepG2 cells were trypsinized and plated to~70% confluence in 96-well plates 24 h before treatment. Prior to the compounds' treatment, the DMEM was removed and replaced with fresh DMEM, and aliquots of the compound stock solutions (500 μM DMSO) were added to obtain final concentrations of 5, 10, 20, 40 and 80 μM. The treated cells were incubated for 24 h at 37 ^oC and under 5% CO_2. Subsequently, the cells were treated with 5 mg/mL MTT (40 μL/well) and incubated for an additional 4 h (37 ^oC, 5% CO_2). Then, DMEM was removed, the formazan crystals were dissolved in DMSO (150 μL/well), and the absorbance at 490 nm was recorded. The cell viability (%) was calculated according to the following equation: cell viability (%) = OD_{490}(sample)/OD_{490}(control) × 100%, where OD_{490}(sample) represents the optical density of the wells treated with various concentration of the compounds and OD_{490}(control) represents that of the wells treated with DMEM + 10% FCS. Three independent trials were conducted, and the averages and standard

deviations are reported. The reported percent cell survival values are relative to untreated control cells.

Microscopy

Cells were seeded in glass-bottom 24-well plate a density of 1×10^4 cells per well and grown for 96 hours. For live cell imaging cell cultures were incubated with **DTPP-ZnCl$_2$**, **DTPP-ZnBr$_2$** and **DTPP-ZnI$_2$** (1% DMSO: 99% cell media) at concentrations 1 μM and maintained at 37°C in an atmosphere of 5% CO_2 and 95% air for incubation times for 60 min. The cells were then washed with PBS (3 × 1 ml per well) and 1 ml of PBS was added to each well. The cells were imaged using upright confocal laser scanning microscopy LSM 710, using 63X and 100X oil immersion lens. Excitation energy of 820 nm was used and the fluorescence emission measured at 495-582 nm. For Syto9, excitation energy 488 nm was used and fluorescence emission was measured at 500–550 nm. Nuclear staining was performed using DAPI (500 nM) for 10 min in PBS, excitation energy 405 nm was used and fluorescence emission was measured at 420–450 nm. TEM samples were sectioned in Araldite resin by microtome and examined on a FEI Tecnai instrument operating at 80 kV equipped with a Gatan 1 k CCD Camera. Image data acquisition and processing was performed using Zeiss LSM Image Browser, Zeiss LSM Image Expert and Image J.



Scheme S2. Schematic illustration of upconversion (a) and two-photon absorption (2PA) process (b). In the upconversion process, the molecules absorb two or more photons subsequently (ground

state (E_0) → excited intermediate state (E_1) → excited state (E_2) in lanthanide-based and triplet-triplet annihilation-based materials). 2PA process involves the simultaneous absorption of two photons from ground state (E_0) to excited state (E_1) in nanoparticals, metal complexes, organic molecules et. al.. During the 2PA process, a non-stationary (virtual) state, which does not exist indeed, will emerge (illustrated by the dashed line in Scheme S2b) due to the interaction of photons and molecules. Hence, upconversion process is like a second-order elementary reaction, which has a higher degree of probability than the case of absorption of two photons simultaneously [1-4].

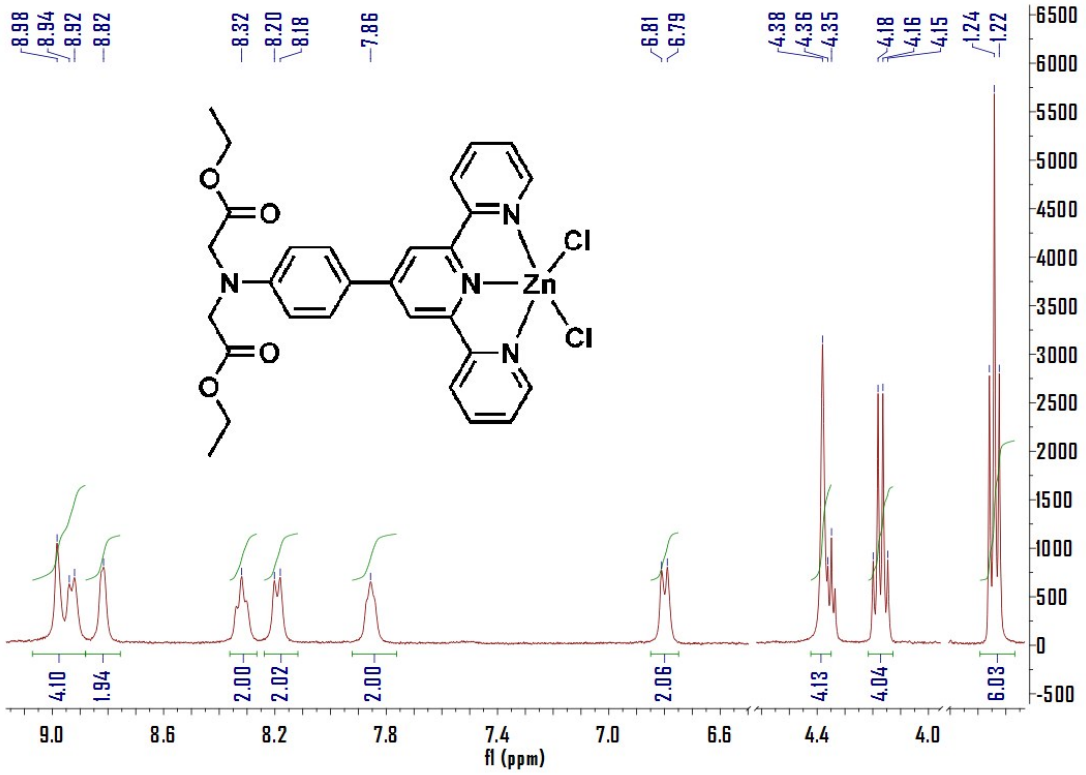


Fig. S1 ^{1}H-NMR spectra of DTPP-$ZnCl_2$.

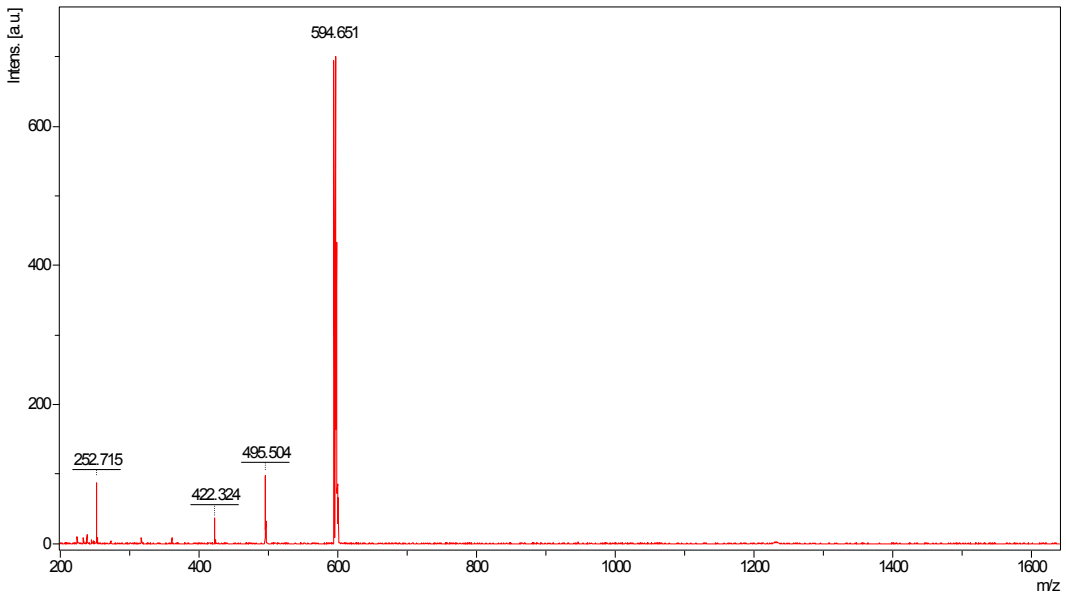


Fig. S2 Mass spectra of DTPP-$ZnCl_2$.

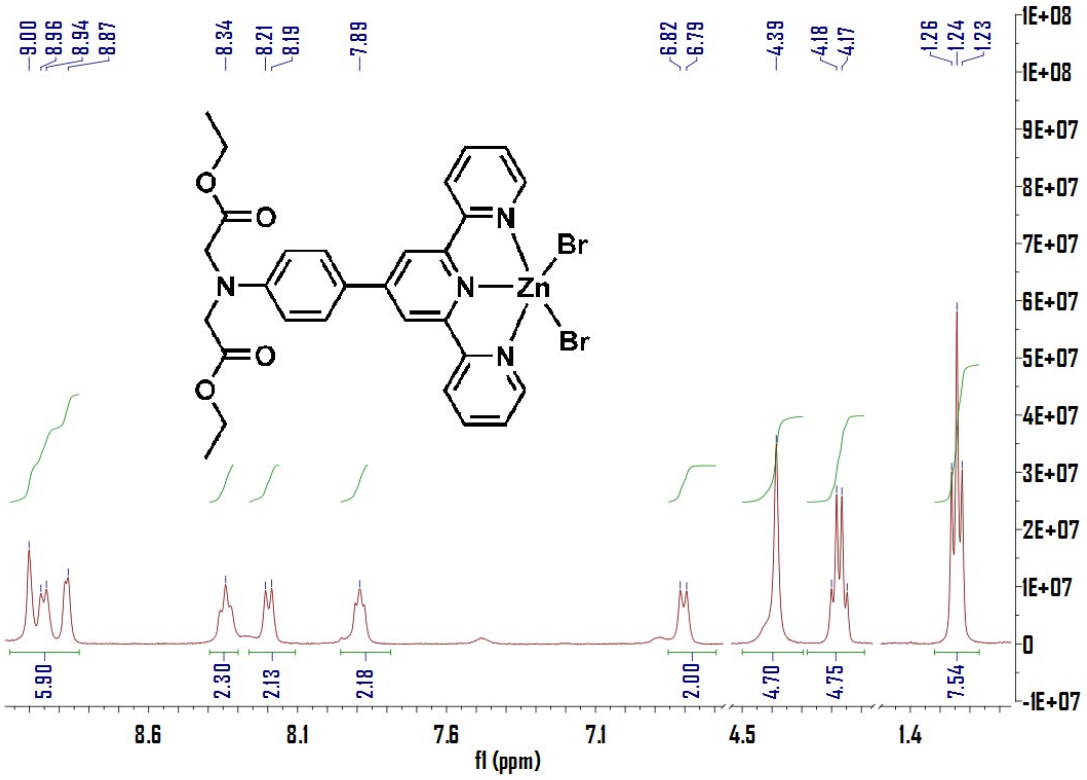


Fig. S3 ^{1}H-NMR spectra of DTPP-$ZnBr_2$.

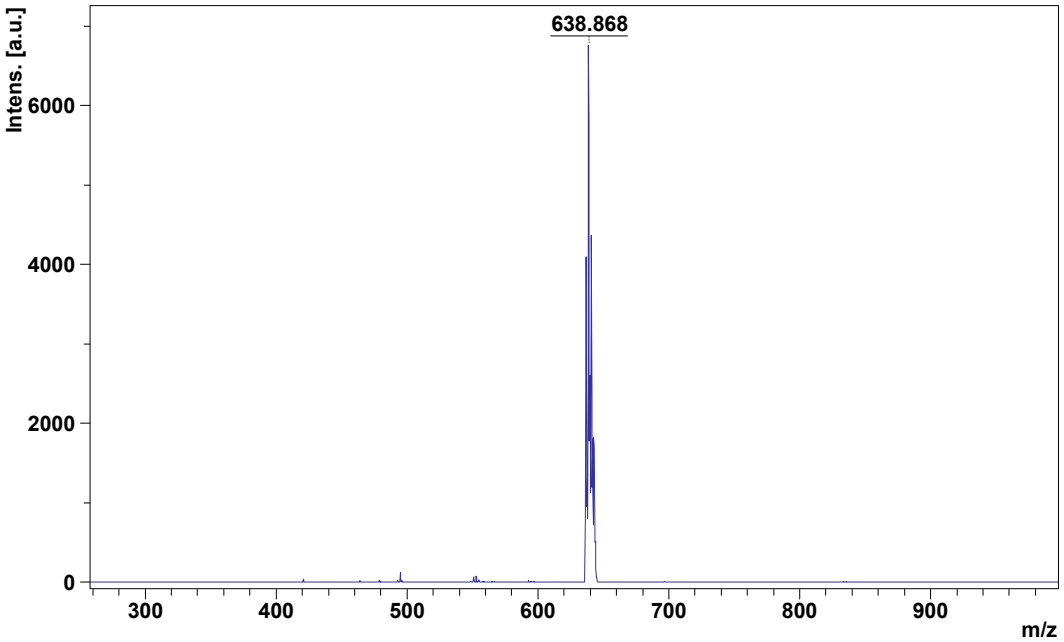


Fig. S4 Mass spectra of DTPP-$ZnBr_2$.

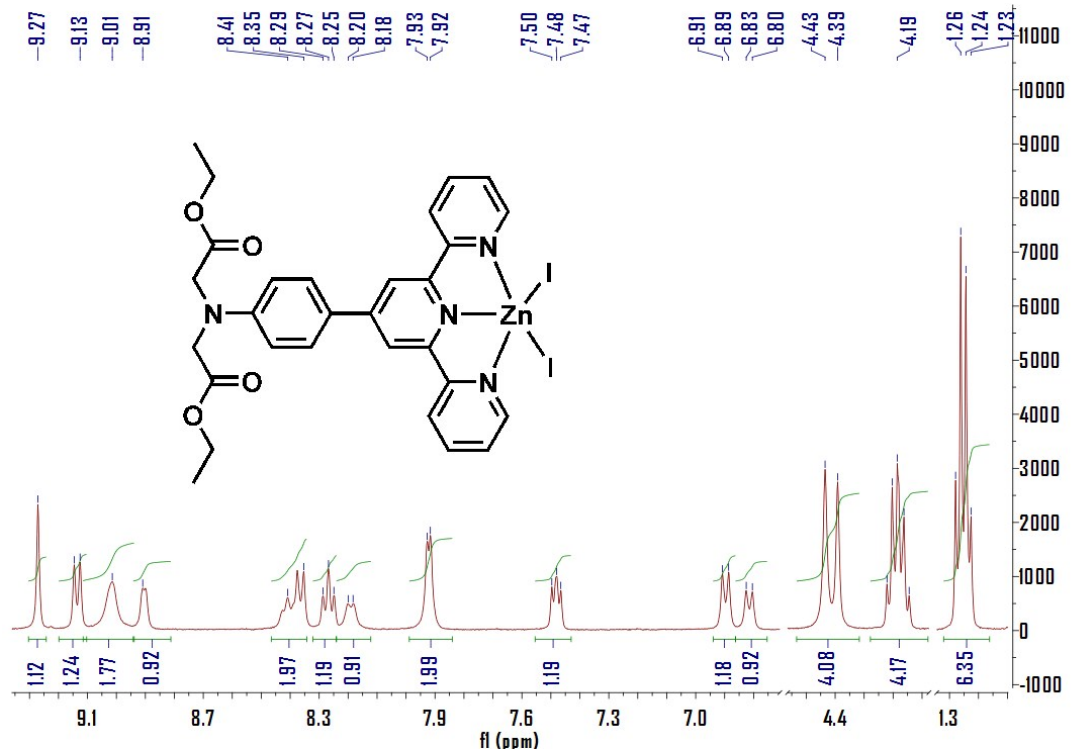


Fig. S5 ^{1}H-NMR spectra of DTPP-ZnI_2.

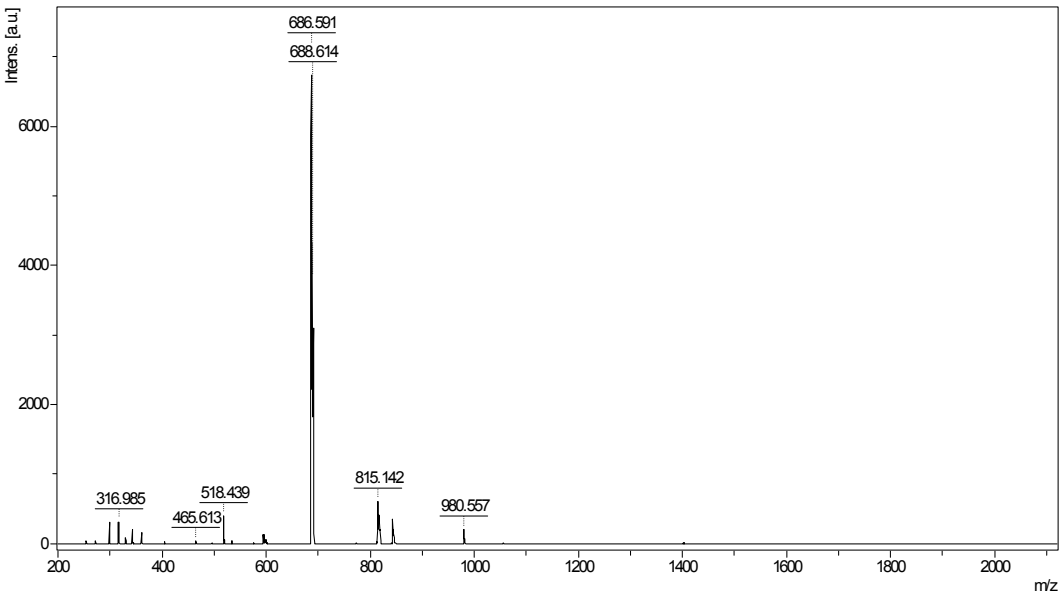


Fig. S6 Mass spectra of DTPP-ZnI_2.

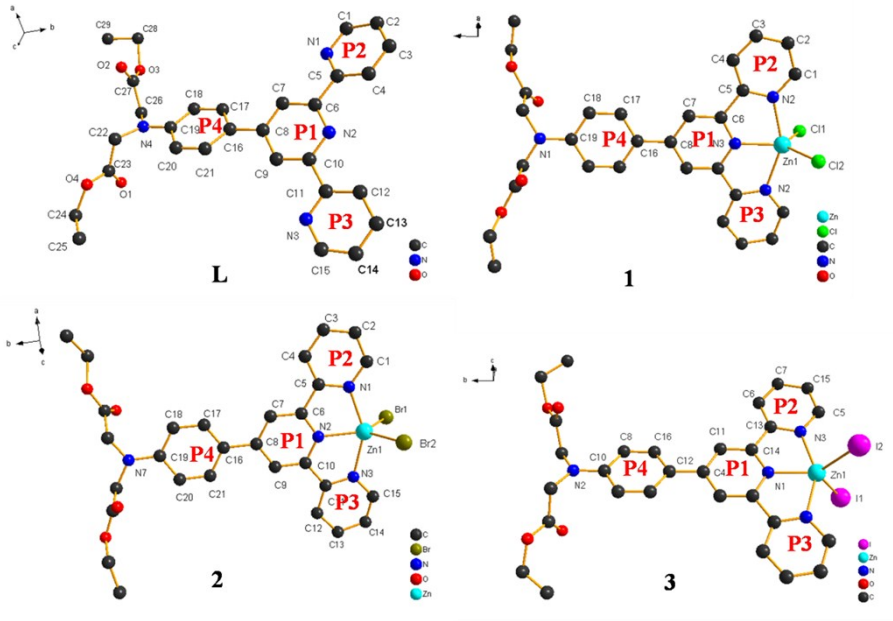


Fig. S7 The single crystal sturcutre of **DTPP-$ZnCl_2$, DTPP-$ZnBr_2$ and DTPP-ZnI_2**

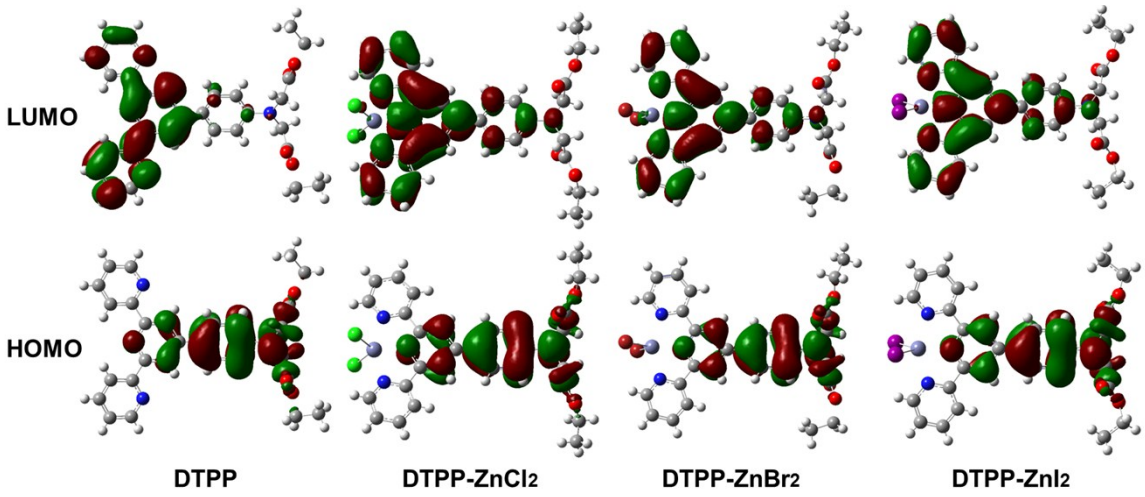


Fig. S8 Electronic cloud distributions of DTPP and its three zinc complexes.

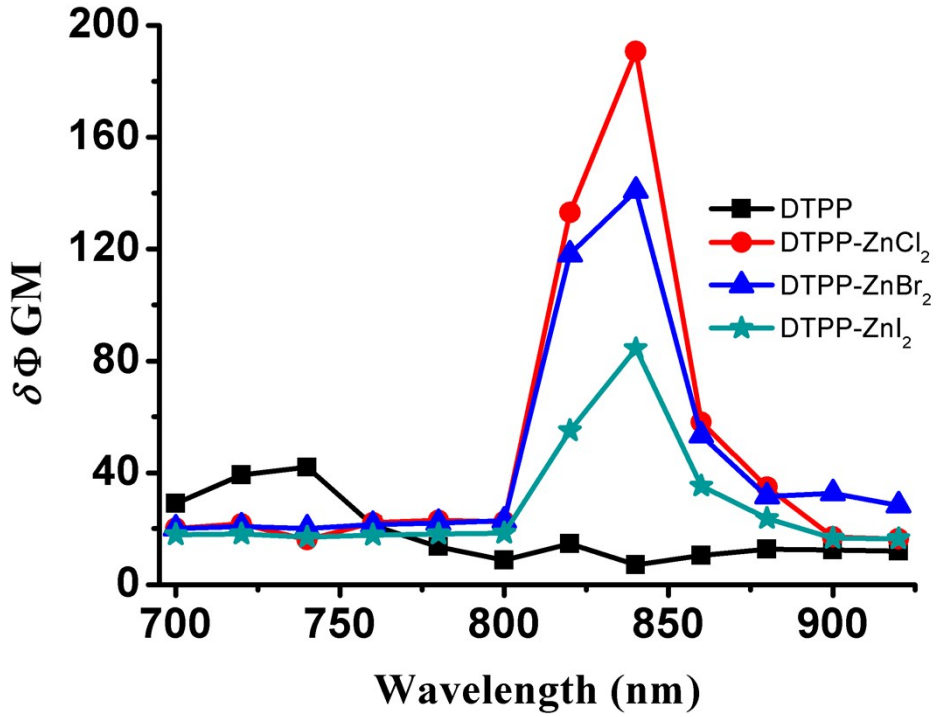


Fig. S9 Two-photon action cross section of **DTPP**, **DTPP-$ZnCl_2$, DTPP-$ZnBr_2$ and DTPP-ZnI_2** in DMF with concentration 5.0×10^{-4} mol/L.

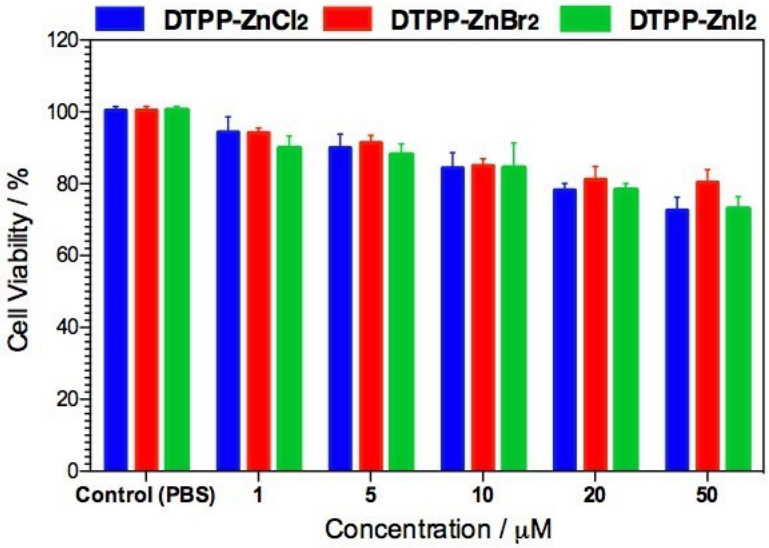


Fig. S10 Cell viabilities of DTPP-$ZnCl_2$, DTPP-$ZnBr_2$ and DTPP-ZnI_2 by MTT assays.

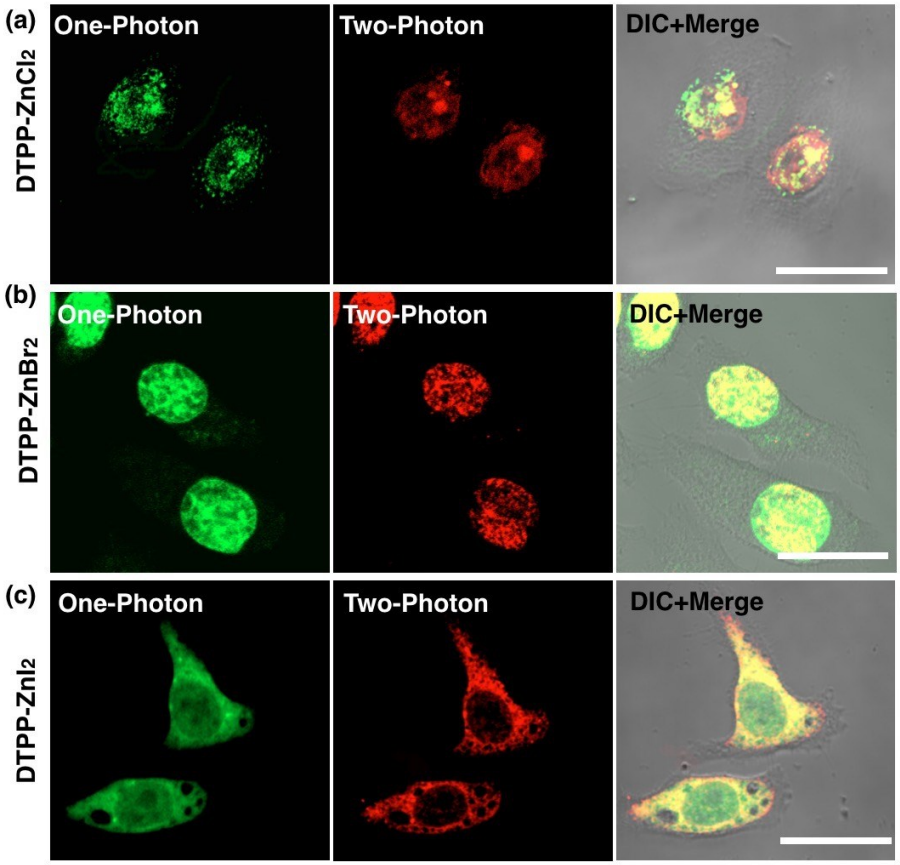


Fig. S11 One- and two-photon fluorescent images of HepG2 cells incubated in DTPP-$ZnCl_2$, DTPP-$ZnBr_2$ and DTPP-ZnI_2.

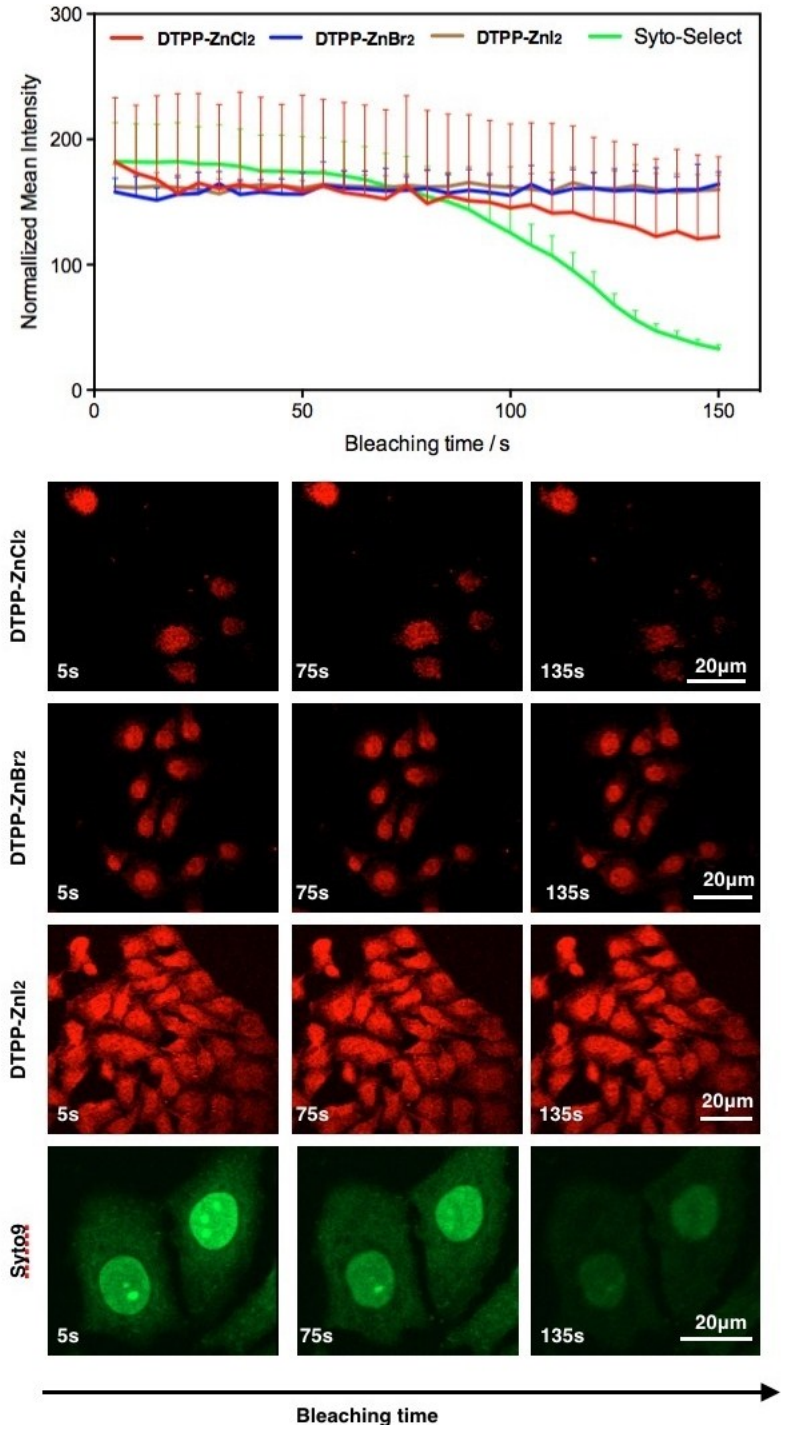


Fig. S12 The photon bleaching profile of complexes DTPP-$ZnCl_2$, DTPP-$ZnBr_2$ and DTPP-ZnI_2 in HepG2 cells under continues two-photon irradiation.

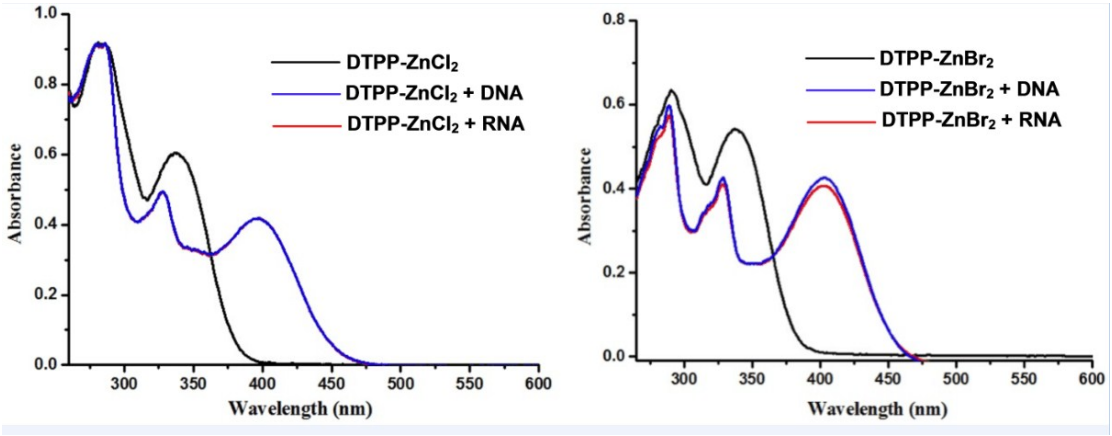


Fig. S13 Absorption spectra of DTPP-$ZnCl_2$ and DTPP-$ZnBr_2$ with adding of RNA and DNA.

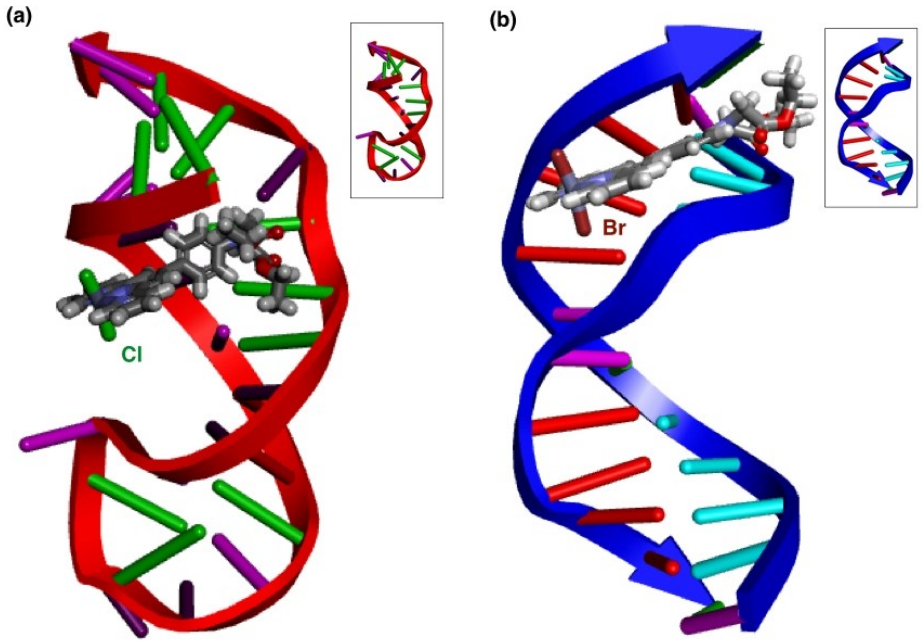


Fig. S14 Models obtained after molecular modeling of the interaction of **1** and **2** with (a) RNA and (b) DNA fragment, respectively. Inset: the structure of the corresponding RNA and DNA fragment

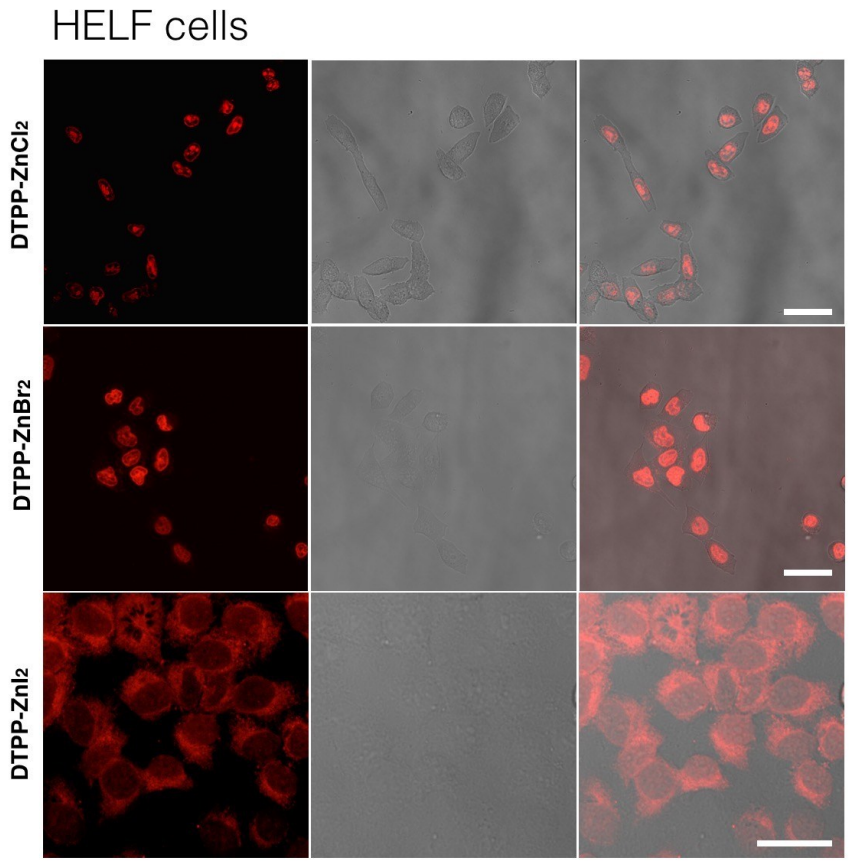


Fig. S15 Complexes DTPP-$ZnCl_2$, DTPP-$ZnBr_2$ and DTPP-ZnI_2 internalized with HELF cells. The scale bars represent 20μm.

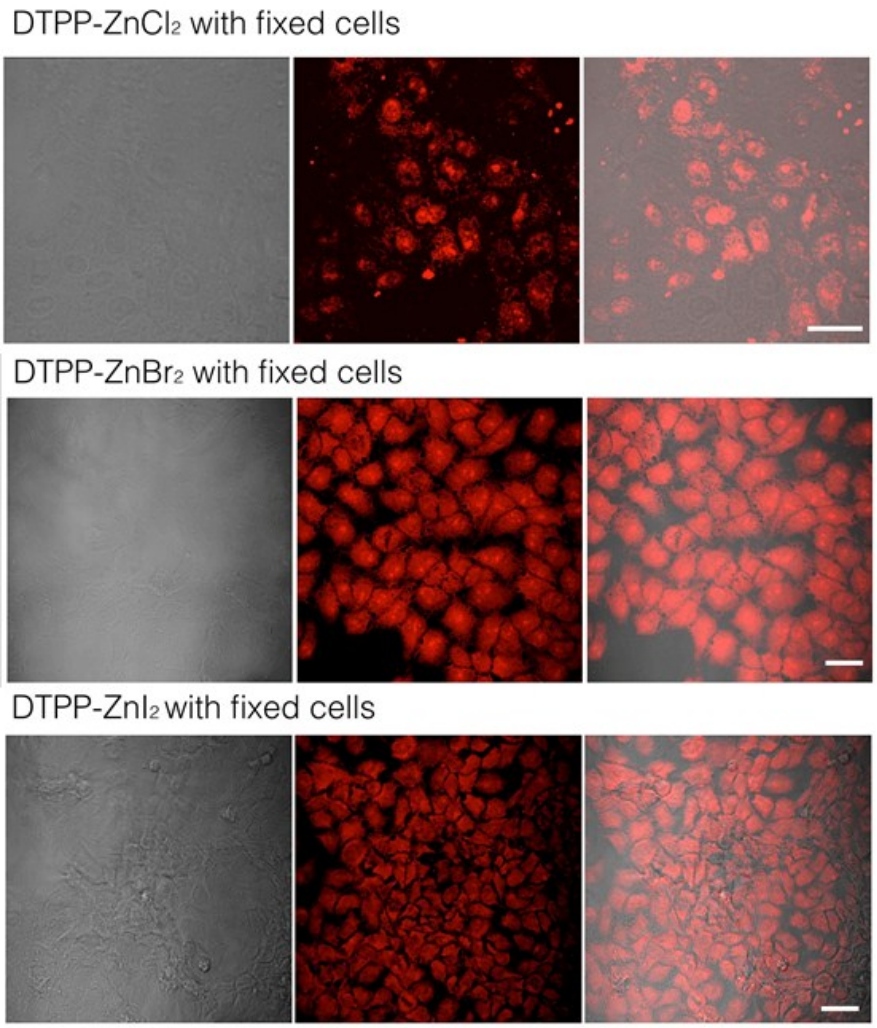


Fig. S16 Complexes DTPP-$ZnCl_2$, DTPP-$ZnBr_2$ and DTPP-ZnI_2 internalized with pre-fixed HepG2 cells. The scale bars represent 20 μm.

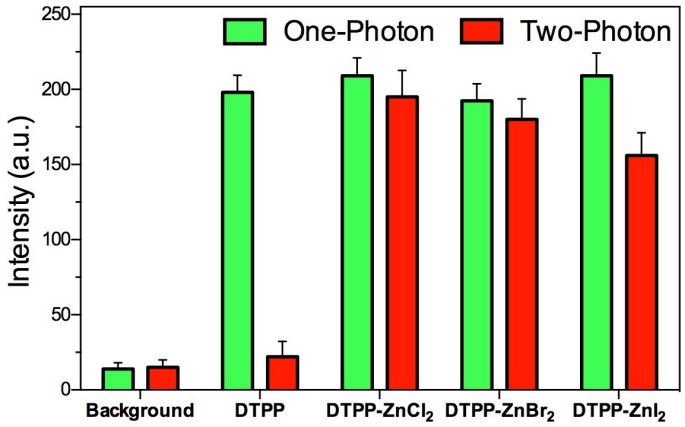


Fig. S17 One- and two-photon fluorescence intensities of DTPP-$ZnCl_2$, DTPP-$ZnBr_2$ and DTPP-ZnI_2 in HepG2 cells.

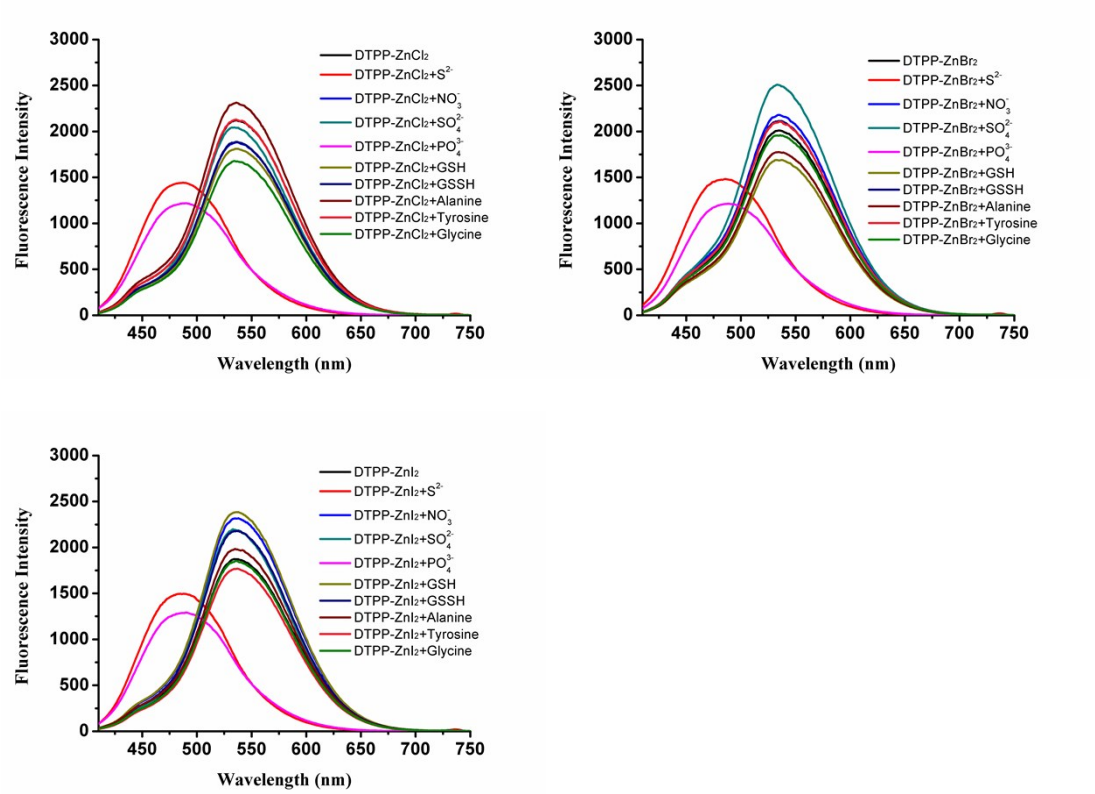


Fig. S18 The emission spectra of Zn(II) complexes mixed with different anions and bioions in PBS/acetonitrile (v/v = 2:1)

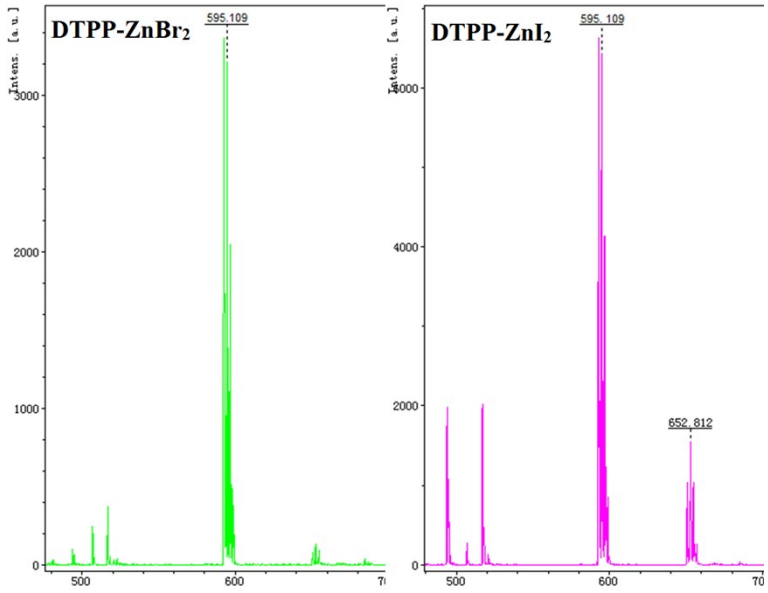


Fig. S19 The mass spectra of DTPP-$ZnBr_2$ and DTPP-ZnI_2 dissolved in DMEM/acetonitrile (v/v=2:1)

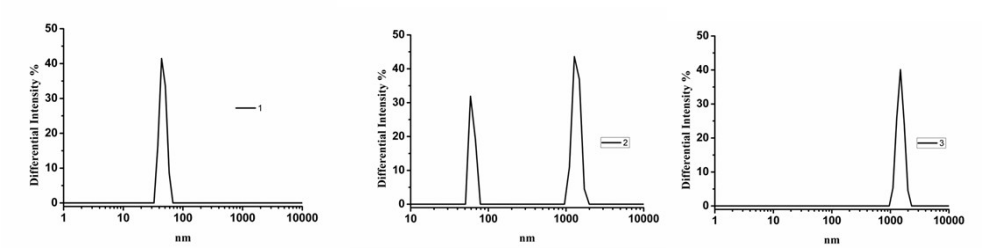


Fig. S20 The DLS spectra of DTPP-$ZnCl_2$, DTPP-$ZnBr_2$ and DTPP-ZnI_2 dissolved in DMEM cell culture medium.

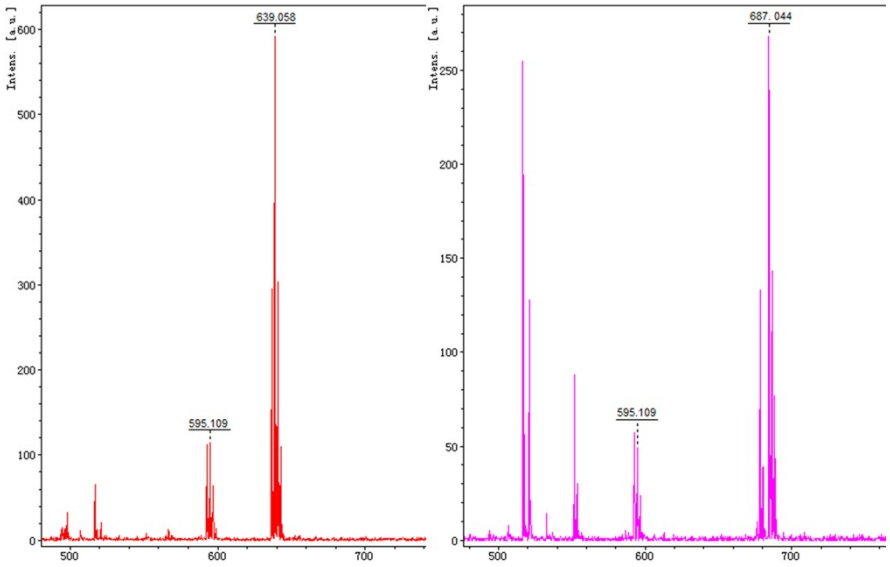


Fig. S21 The mass spectra of DTPP-$ZnBr_2$ (left) and DTPP-ZnI_2 (right) dissolved in DMEM cell culture medium for 24 h.

Reference:

[1] X. Zhu, Q. Su, W. Feng, F. Li, *Chem. Soc. Rev.*, **2017**, 46, 1025-1039.
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