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Electronic Supporting Information (ESI):

Targeted Copper Supplementation Oriented Theranostic for Fluorescence and ^{19}F NMR Detection of Tumor

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General

$\mathbf{F_{465}}$ (1 mM), $\mathbf{F_{508}}$ (1 mM), $\mathbf{F_{542}}$**-Cu** (1 mM) and **M985** (1 mM) were dissolved in deionized water and stored at 4 °C for use. Lysosome Isolation Kit was purchased from ToYongBio (Shanghai, China). Streptavidin-coated magnetic beads were purchased from PangoBio (Nanjing, China). Hoechst33342, ER-Tracker, Dihydroethidium (DHE), propidium iodide (PI), Lyso-Tracker, Mito-Tracker and Golgi-Tracker were purchased from Keygen Biotech (Nanjing, China).

Fluorescence spectra were obtained on a Hitachi F-7000 fluorescence spectrometer. NMR spectra were acquired on a Bruker DRX-500 spectrometer with TMS as the internal standard. Confocal fluorescence imaging was performed with a ZEISS Laser Scanning Microscope (Zeiss LSM 710). Mass spectrometry spectra were determined on Thermo LCQ FLEET. Inductively coupled plasma optical emission spectrometry (ICP-OES) was acquired on Optima 5300DV Series. In vivo imaging was conducted with Lumina XRMS III. Preparative Agilent 1260 Infinity II HPLC was used to purify compounds. Lauda E100 circulating water pump was employed to maintain constant temperature at 37 °C. Ultrapure water were prepared from Milli-Q A10 system. All of the pH solutions were measured with JENCO 6230 M pH meter.

Synthesis of F_{542}

F_{465}, F_{508}, Compound **1**, Compound **2** were synthesized as previously reported literatures.[1,2,3] Compound **1** (1.680 g, 8.155 mmol) and Compound **2** (300 mg, 0.806 mmol) were added to 60 ml of chloroform in the flask, and dropped with triethylamine (96 mg, 0.950 mmol). The above solution was stirred for 18 h under nitrogen atmosphere at room temperature. After that, the solution was concentrated under reduced pressure, the obtained residue was re-dissolved in DCM and washed with brine three times. The organic solution was dried over anhydrous $MgSO_4$. The crude product was purified by silica gel column chromatography (chloroform: methanol: ammonium hydroxide (10: 1: 0.05 v/v/v)) to afford a red solid (297 mg, 68 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, J=7.7, 1H), 7.49 (d, 2H), 7.20 (d, J=7.6, 2H), 7.01 (d, J=7.5, 1H), 6.87 (d, J=7.5, 1H), 5.97 (s, 2H), 4.05 (d, J=15.9, 4H), 3.80 (s, 2H), 3.15 (s, 2H), 2.99 (s, 2H), 2.56 (s, 6H), 2.39 (s, 4H), 1.82 (s, 2H), 1.33 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 160.86, 158.91, 155.31, 143.06, 136.75, 133.68, 131.45, 129.56, 127.88, 121.12, 119.99, 61.67, 59.49, 53.54, 48.66, 48.08, 47.32, 29.67, 14.53, 14.40. ESI-MS (methanol): Calcd. $\mathbf{F_{542}}$ $[M+H]^+$: 543.32, found 543.32.

Synthesis of Biotin-His[3]

Histidine (150 mg 0.968 mmol) and NHS-Biotin (280 mg, 0.821 mmol) were dissolved to 10 ml of DMF in the flask under nitrogen. The above solution was stirred at room temperature for 18 h. After the solution was removed under vacuum, the residue was re-dissolved in deionized water and filtrated to afford clear solution. The solution was lyophilized and then separated by silica gel column chromatography (Ethyl acetate: Methanol (10: 3 v/v)) to obtain a white solid (280 mg, 88 %). ESI-MS (H_2O): Calcd. **Biotin-His** $[M+H]^+$: 382.15, found 382.15.

Synthesis of F_{542}-Cu

F_{542} (200 mg, 0.370 mmol) was dissolved in 5 ml of ethanol in the flask, and 1 ml of $CuCl_2 \cdot 2H_2O$ (75.50 mg, 0.450 mmol) ethanol solution was dropped subsequently, accompanied with the formation of deep red precipitation. The mixture was stirred at room temperature for 6 hours under nitrogen atmosphere. The deep red precipitation was filtered and washed three times with ethanol and dried under vacuum (233 mg, 93 %). ESI-MS (H_2O): Calcd. for **F_{542}-Cu**: 663.20, found 663.20.

Synthesis of M985

F_{542}-Cu (50 mg, 0.074 mmol) was dissolved in 5 ml of deionized water in the flask, subsequently, the Biotin-His (34 mg, 0.089 mmol) was added. The above mixture was stirred at room temperature for 12 hours under nitrogen atmosphere. The final reaction solution was purified to homogeneity by preparative HPLC and then lyophilized to obtain **M985** as a white solid (49 mg, 65%). ESI-MS (H_2O): Calcd. For **M985**: 985.39, found 985.38.

Determination of quantum yields

Quantum yields were determined at 25 °C, Fluorescein (φ = 0.90) in 0.1 M NaOH was employed as a standard substance. The absorption of Fluorescein was adjusted to the same value (abs < 0.1) as that of fluorescent compounds. Excitation wavelength was chosen at 460 nm; the emission wavelength were corrected and integrated from 480 nm to 650 nm. The quantum yields were calculated with the following equation:

$$\Phi_{sample} = \Phi_{standard} \, (Grad_{sample} / Grad_{standard}) \, (\eta_{sample}^2 / \eta_{standard}^2)$$

Φ is the quantum yield, Grad is the slope of the plot of absorbance versus integrated emission intensity, and η is the adopted refractive index of the solvent.[4]

Cell viability assay

HepG-2, HeLa, A549, A549cisR, MCF-7, LO2, WI-38 cells were cultured in a humidified incubator, which provided an atmosphere of 5 % CO_2 and 95 % air at 37 °C. The culture medium were DMEM (Dulbecco's modified Eagle's medium, Gibco BRL) and RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) medium, which contained 10 % FBS (fetal bovine serum, Gibco BRL), 100 μg/mL streptomycin (Gibco BRL), and 100 U/mL penicillin (Gibco BRL). The inhibitory concentration of the compounds toward different cell lines was evaluated by MTT assay. Cells were seeded in a 96-well plate at 5×10^3 cells/well and allowed to grow 24 h prior to exposure to different concentrations of tested compounds for further 48 h. 20 μL of MTT solution/per well (5 mg/mL) was added and the cells were incubated for further 4 h, DMSO (150 μL/well) was then added for 10 min after removing the total medium. The absorbance at 490 nm was collected using a Varioskan Flash microplate reader.

Transmission electron microscopy

HepG-2 cells were treated with dithiothreitol (4 mM), F_{542} (10 μM), **M985** (10 μM) at 37 ºC for 12 h. After that, cells were collected and fixed overnight at 4 ºC in PBS (pH 7.4), which contained 2.5 % glutaraldehyde. Subsequently, the cells were treated with OsO_4 (osmium tetroxide), stained with uranyl acetate and lead citrate. The cells were visualized using a transmission electron microscope (JEM 100 CX, JEOL, Tokyo, Japan), and the images were photographed by the Eversmart Jazz program (Scitex).

Western blot analysis

HepG-2 cells were seeded in 6-well plates and cultured for 24 h under 21 % pO_2, and then exposed to varied concentrations of F_{542} or **M985** for 24 h. After that, cells were collected and washed with ice-cold PBS (pH 7.4) three times. The extracts of total cellular protein was obtained at 4 °C in lysis buffer

containing 20 mM Tris-HCl (pH 8.0), 250 mM NaCl, 0.4 mM Na_3VO_4, 1 % SDS and 1×Complete mini protease inhibitor cocktail tablets. Samples were separated by 12 % SDS-PAGE and transferred to an immobilon-P transfer membrane (Millipore, USA). Membranes were blocked with 5% non-fat milk in TBS containing 0.1 % Tween-20 at room temperature for 1 h, and incubated with primary antibodies. The antibodies were diluted in TBS with 5% non-fat milk overnight at 4 °C. Then the blots were incubated with an HRP-conjugated anti-rabbit secondary antibody (1:4000) and an anti-mouse secondary antibody (1:4000) for 1 h at room temperature, respectively. The data were captured on the enhanced chemiluminescence (ECL, Millipore).

Co-localization assay

HepG-2 cells were incubated with F_{542} (1 μM) at 37 °C for 0.5 h and further co-incubated with Mito Tracker Red CMXRos (100 nM), Lyso Tracker DND-99 (100 nM), ER Tracker Red (100 nM), Golgi Tracker Red (1 mM), Hoechst33342 at 37 °C for 1 h. After that, the cells were washed thrice with ice-cold PBS, and imaged by laser confocal microscopy (LSM 710, Carl Zeiss). Images were collected by using green channel: λ_{ex}= 488 nm, λ_{em}= 490-550 nm; red channel: λ_{ex}= 543 nm, λ_{em}= 570-650 nm; blue channel: λ_{ex}= 405 nm, λ_{em}= 410-460 nm.

Flow Cytometry

HepG-2 cells were plated into flat-bottomed culture dishes (Φ 60 mm) containing 5 mL of DMEM. After incubation (37 °C, 5 % CO_2) for 2 days, the media was replaced with fresh DMEM, and F_{542} were added for different time spans. The control group was treated with F_{542} for 20 min. PI or DHE was stained for 0.5 h at 37 °C before FCM analysis. For cell imaging assay, the HepG-2 cells were treated with F_{542}-Cu (1 μM) and **M985** (1 μM) for 24 h, respectively. The cell samples were illuminated at 488 nm on a BDLSR Fortessa flow-cytometer. The fluorescence of the forward-scattered and side-

scattered light from 1×10^4 cells were detected at rate of 150 events/s. The flow cytometry data were analysed using Flow-Jo software.

Lysosome Isolation assay and LC-MS Analysis

HepG-2 cells were seeded in the flat-bottomed culture dishes (Φ 100 mm) containing 10 mL of DMEM. After incubation (37 °C, 5 % CO_2) for 1 day, the media was replaced with fresh DMEM, and **M985** (20 μM) was added and further incubated for 12 h. After that, the cells were harvested and washed with ice-cold PBS twice. And then the cells were re-suspended with ice-cold PBS (pH 7.4), counted and centrifuged for 5 min under 4 °C at 600 g, and the supernatant was discarded. The packed cell volume maintained up to be 2.0 ml. The next procedures were strictly followed as the technical bulletin of Lysosome Isolation Kit (Sigma-Aldrich). The isolated lysosomes of HepG-2 cells were subsequently crushed in ice-bath by ultrasonic to afford clear solution. And then the above solution was added methanol and centrifuged at 5000 g for 20 min under room temperature, the supernatant was collected for LC-MS analysis. HPLC analysis used a linear gradient from 40 % methanol/ 60 % H_2O to 80 % methanol/20 % H_2O over 10 min using Thermo LCQ Fleet, C8, 5μm, 2.1×150 mm column.

Cell Uptake Assay

HepG-2 cells and WI-38 cells were co-incubated with F_{542}-Cu (5 μM) and **M985** (5 μM) for 24 h, respectively. For the control group, cells were cultivated without any copper complex. The treated cells were collected and washed with ice-cold PBS (pH 7.4) thrice and re-suspended with ice-cold PBS. And then the cells were counted by a portable cell counter. The packed cells were digested by water (50 μl), concentrated nitric acid (70 μl) at 95 °C for 1 h, hydrogen peroxide (30 %, 20 μl) at 95 °C for 1 h, and concentrated hydrochloric acid (35 μl) at 37 °C for 0.5 h, successively. The digested solution was diluted to 1 ml

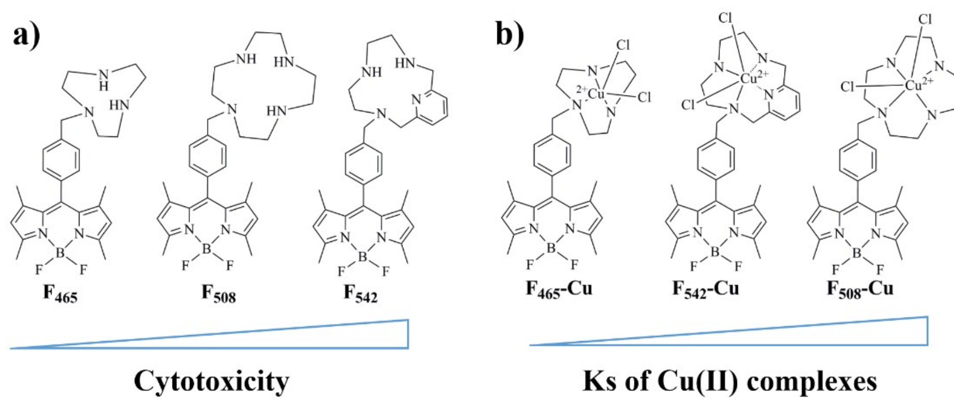
with deionized water and total cellular copper concentration was determined using ICP-OES.

***In vivo* evaluation**

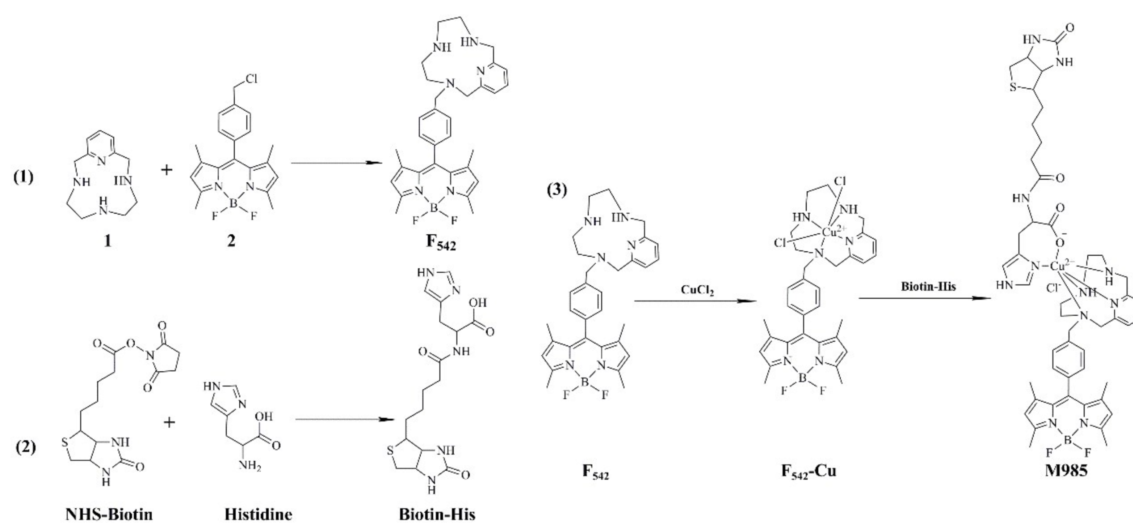
Specific pathogen-free (SPF) female BALB/c mice (four weeks old), were purchased from Model Animal Research Center of Nanjing University and bred in an axenic environment. All animal operations were in accord with institutional animal use and care regulations approved by the Model Animal Research Center of Nanjing University. Hepatic carcinoma tumor-bearing model was established by subcutaneous injection of HepG-2 cells (1×10^6) into the selected positions of the nude mice. The volume of tumor was calculated using the following formula: tumor volume = length × width^2 × 0.5. The tumor-bearing mice were weighed and randomly divided into four groups when the tumor volume reached up to 30 mm^3, and subjected to the following administration: 1. Saline; 2. 5 mg cisplatin/Kg body weight; 3. 5 mg **M985**/Kg body weight. The saline, cisplatin and **M985** were administrated by tail intravenous injection, successive medication for 16 days, and meanwhile the tumor volumes and body weights were measured. At day 17th, the mice were sacrificed to collect tumors, and then tumors were weighed, washed with saline thrice and fixed in the 10 % neutral-buffered formalin.

H&E Staining

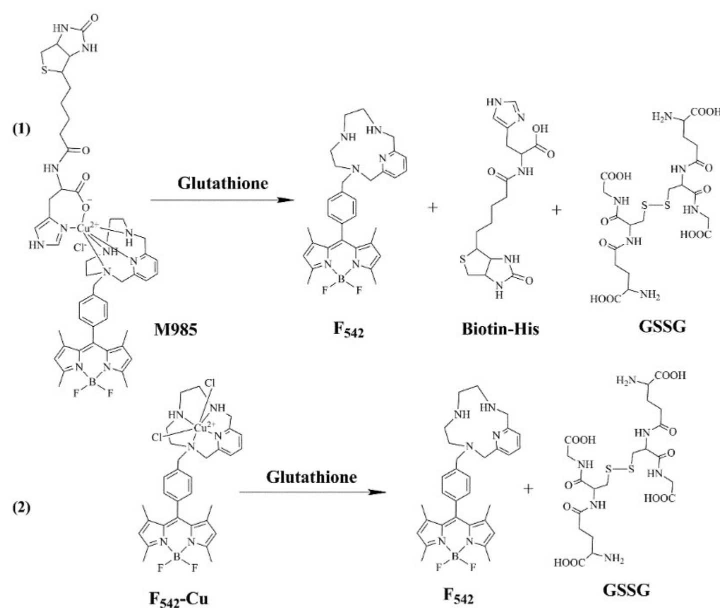
H&E staining was carried out according to a protocol provided by the vendor (BBC Biochemical). Firstly, 8 μm of tumor cryogenic slides were prepared and fixed with 10 % formalin for 30 min at room temperature. After washing with running water for 5 min, the slides were treated with gradient concentrations of alcohol (100 %, 95 % and 70 %), each for 20 s. The hematoxylin staining was performed for 3 min and washed with water for 1 min. The eosin staining was performed for 1 min. The slides were washed, treated with xylene, and mounted with Canada balsam. The images were captured using a Nikon Eclipse 90i.



Scheme S1. The structures of BODIPY-armed azamacrocyclic compounds and copper complexes.



Scheme S2. Synthesis procedure of F_{542}, Biotin-His and copper vehicle **M985**



Scheme S3. Proposed reaction mechanisms of copper vehicle **M985** and F_{542}-Cu with GSH.

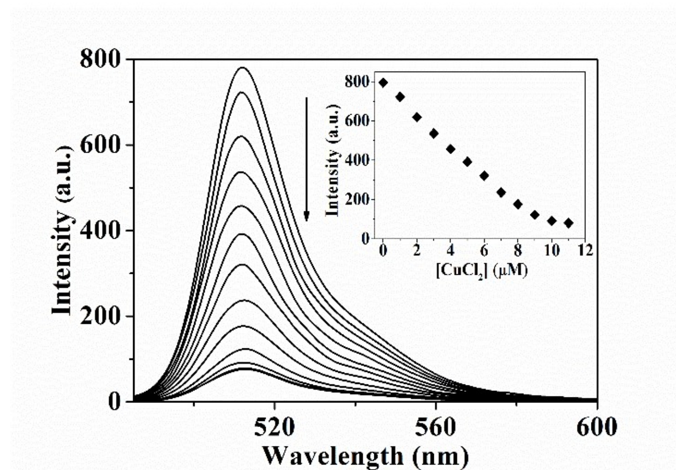


Figure S1. Fluorescence spectra of F_{542} (10 μM) in Tris-HCl solution (20 mM, pH 7.4) with the titrations of $CuCl_2$. Inset: Changes of the fluorescence intensity at 510 nm upon the titration. All measurements were taken at 37 °C, slit width 2.5 nm. Excitation wavelength 470 nm. The spectra were recorded 30 min after adding $CuCl_2$.

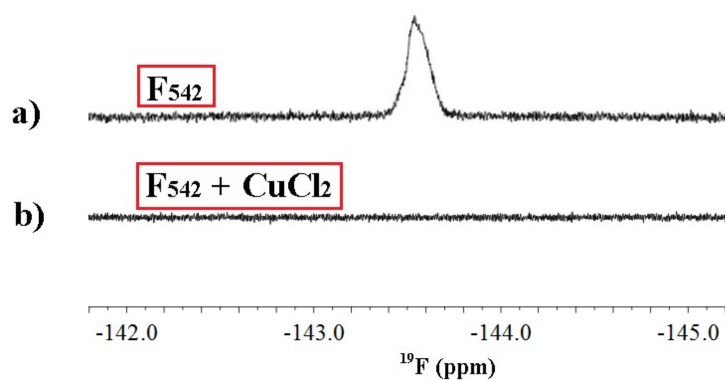


Figure S2. (a) ^{19}F NMR spectra of F_{542} (100 μM) in Tris-HCl buffer (20 mM, pH 7.4, D_2O); (b) ^{19}F NMR of F_{542} (100 μM) in Tris-HCl buffer (20 mM, pH 7.4, D_2O) after addition of $CuCl_2$ (100 μM) for 30 min. The ^{19}F NMR scans 1024 times for each measurement.

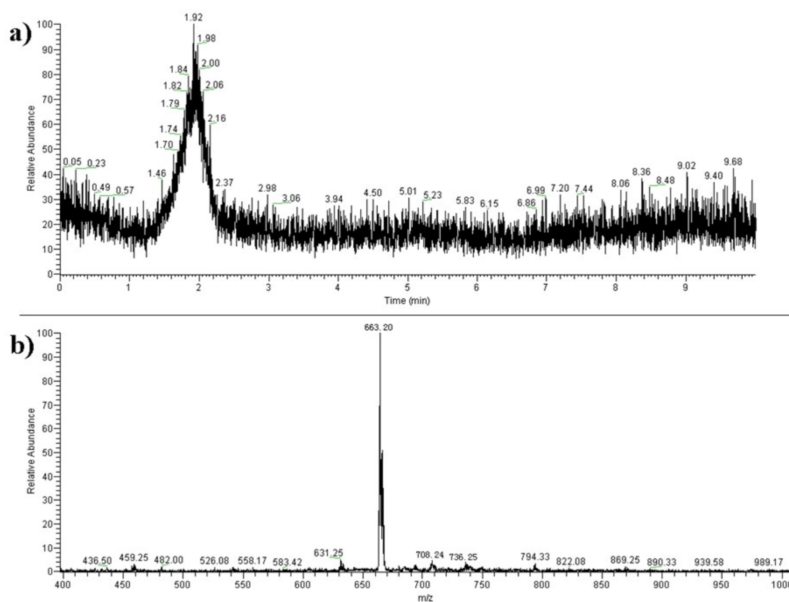


Figure S3. LC-MS analysis for the F_{542}-Cu in water. HPLC runs used a linear gradient from 40 % methanol/60 % H_2O to 80 % methanol/20 % H_2O over 10 min using Thermo LCQ Fleet, C8, 5μm, 2.1×150 mm column. (a) Ion flows at different time points. ESI-MS spectra of peaks eluting at (b) 1.92 min during gradient.

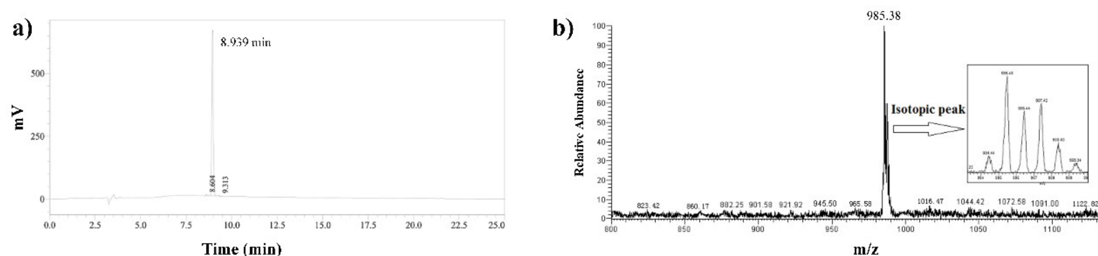


Figure S4. Analytical HPLC trace (220 nm) and ESI-MS spectrum of **M985**. HPLC runs used a linear gradient from 20 % methanol/80 % H_2O to 40 % methanol/60 % H_2O over 25 min using Alltima TM, C18, 4.6×250 mm column. (a) HPLC chromatogram; (b) ESI-MS spectra of **M985**.

In vitro cytotoxicity

Table S1. Cytotoxic effects of the tested compounds toward several human cell lines. Cell viability was assayed using MTT test (48 h). ND: no data. The error represent ± S.D. (n= 3).

Compound	IC$_{50}$ (μM)						
	HepG-2	LO2	WI-38	A549	A549cisR	Hela	MCF-7
M985	2.62±0.15	16.35±1.28	12.17±1.31	4.21±0.36	4.68±0.64	4.03±0.31	3.66±0.32
F$_{542}$	3.98±0.17	3.22±0.24	1.13±0.28	5.93±0.36	5.23±0.72	4.89±0.22	5.73±0.47
F$_{508}$	10.07±0.91	10.83±1.31	9.63±1.14	8.71±0.73	9.10±0.95	8.11±0.64	6.79±0.78
F$_{465}$	16.33±1.61	12.82±1.48	10.86±1.02	20.12±2.05	25.12±3.23	24.35±1.42	18.33±1.65
Sorafenib	24.66±3.12	18.37±1.04	13.45±0.81	19.48±2.17	46.63±3.49	20.6±1.37	28.53±2.41
Gefitinib	30.39±2.42	16.83±1.62	15.36±1.31	25.61±2.48	24.28±2.11	26.35±2.18	23.52±2.97
F$_{542}$-Cu	155.82±8.3	163.52±7.1	ND	ND	ND	ND	ND

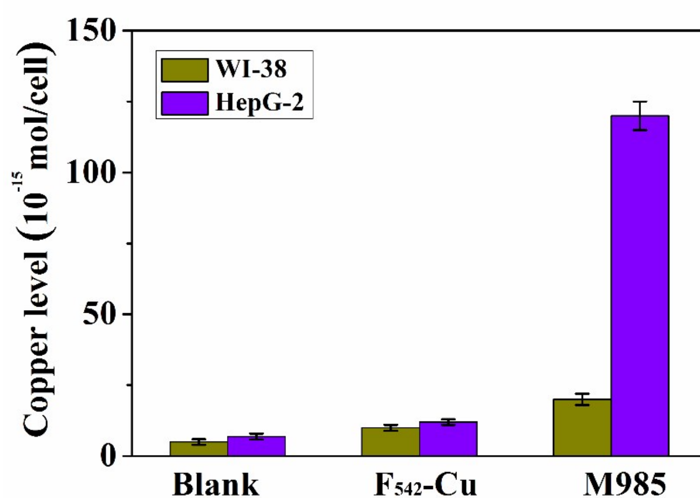


Figure S5. Cellular uptake of copper in HepG-2 cells and WI-38 cells measured by ICP-OES. The data were acquired after cell lines were incubated with F$_{542}$-Cu (5 μM) and **M985** (5 μM) for 24 h, respectively.

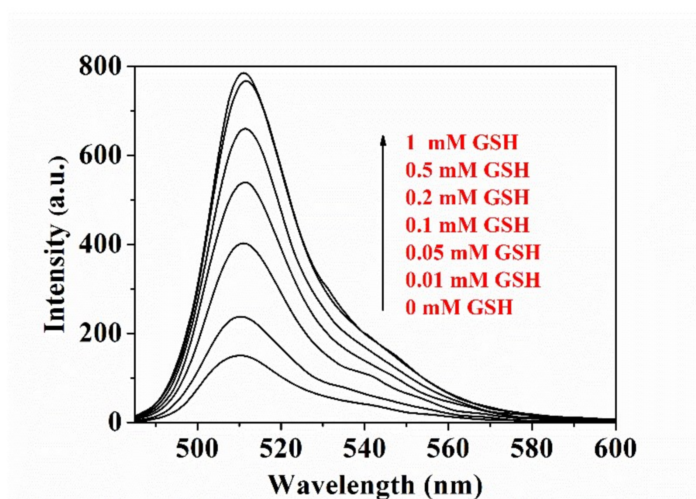


Figure S6. Fluorescence responses of F_{542}-Cu (10 μM) in the presence of varied concentrations of GSH. Fluorescence spectra were recorded 30 min after the addition of GSH. All measurements were taken at 37 °C in Tris-HCl buffer (20 mM, pH 7.4), slit width 2.5 nm. Excitation wavelength 470 nm.

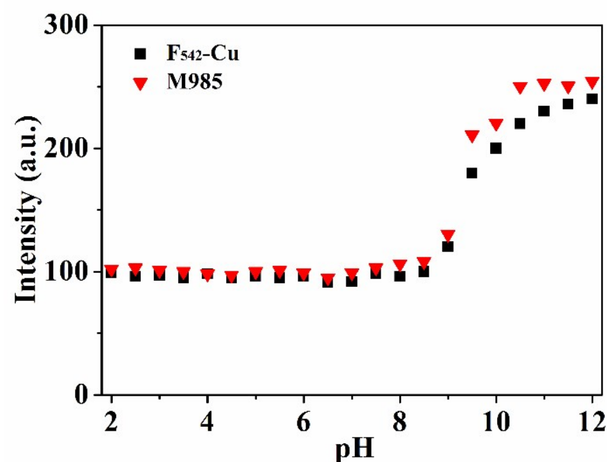


Figure S7. Fluorescence intensity of F_{542}-Cu (10 μM) (black) and **M985** (10 μM) (red) at 510 nm as a function of pH. Slit width 2.5 nm, Ex. 480 nm. All measurements were taken at 37 °C.

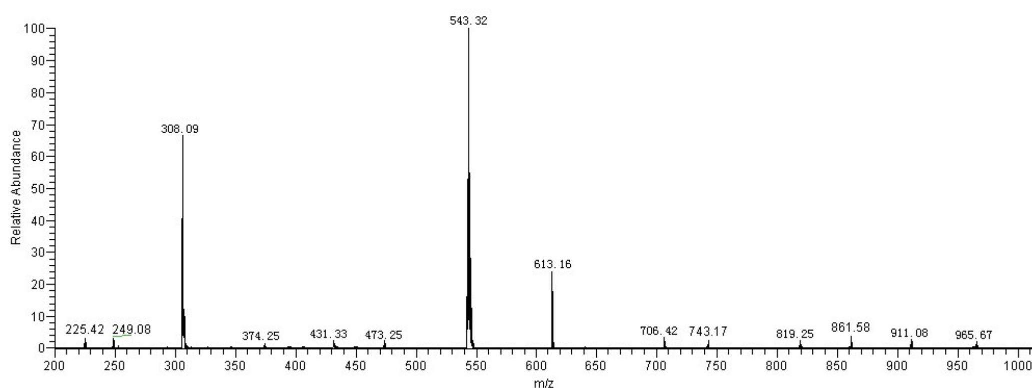


Figure S8. ESI-MS spectrum of F_{542}-Cu in the presence of GSH after 30 min.

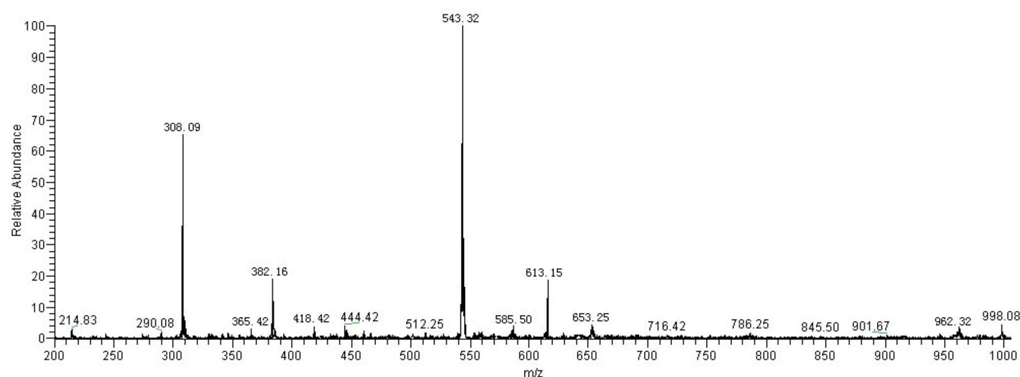


Figure S9. ESI-MS spectrum of **M985** in the presence of GSH after 30 min.

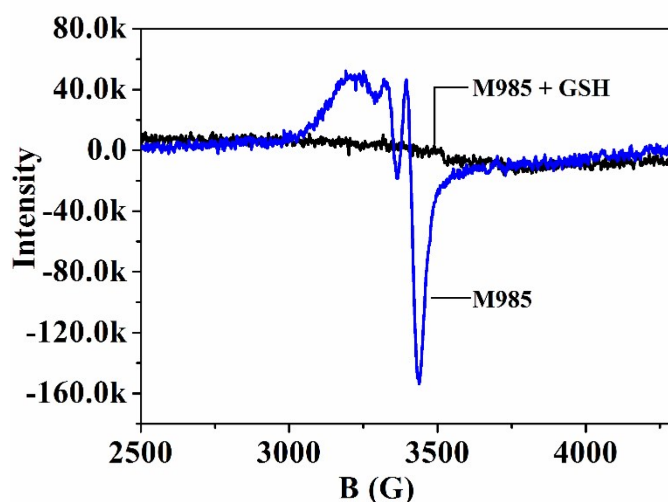
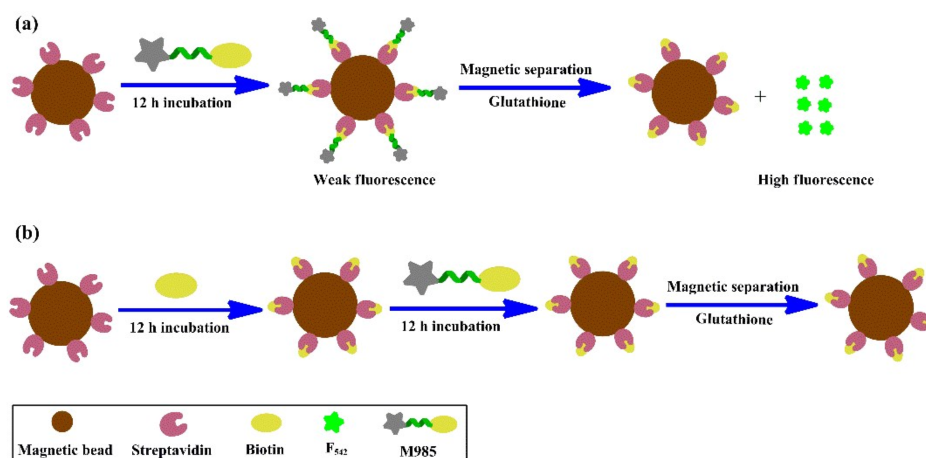


Figure S10. EPR spectra of **M985** (0.1 mM) in the absence and presence of **GSH** (1.0 mM). **Blue line**: The EPR spectra of **M985**; **Black line**: The EPR spectra of **M985** after reacting with **GSH**. The spectra were recorded after 30 min. Study was performed in Tris-HCl buffer (20 mM, pH 7.4) at 37 °C.



Scheme S4. The procedure of biotin-streptavidin binding evaluation. Streptavidin coated magnetic beads were used in this experiment. (a) The F_{542}-Cu (10 μM) and **M985** (10 μM) were incubated with magnetic beads (1 mg/ml, 2 μm in diameter) for 12 h in Tris-HCl (20 mM. pH 7.4) buffer respectively. The beads were separated by magnetic separation and suspended in Tris-HCl buffer, 1mM of GSH was added. The fluorescence spectra were measured after 30 min. (b) The biotin (10 μM) were incubated with magnetic beads (1 mg/ml, 2 μm in diameter) for 12 h in Tris-HCl (20 mM. pH 7.4) buffer. The beads was separated by magnetic separation and suspended, and then **M985** (10 μM) was added and incubated with magnetic beads for 12 h, followed by addition of 1mM GSH. The fluorescence spectra were measured after 30 min.

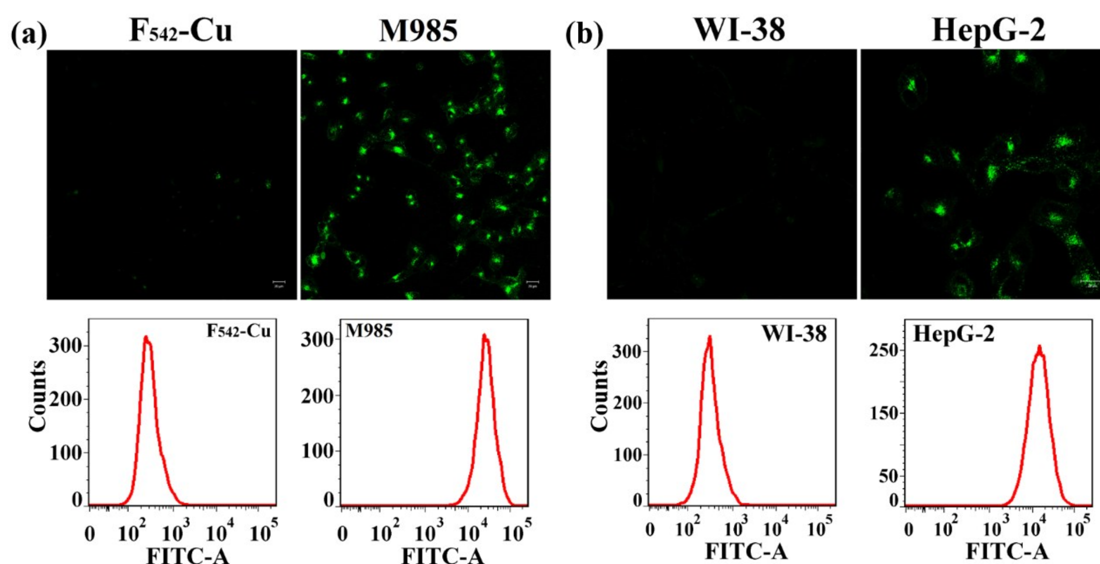


Figure S11. (a) Confocal imaging of HepG-2 cells. The HepG-2 cells were treated with F_{542}-Cu (1 μM) and **M985** (1 μM) for 24 h. Bottom: Flow cytometry analysis of HepG-2 cells stained with F_{542}-Cu and M985. The data were acquired after F_{542}-Cu (1 μM) and **M985** (1 μM) were incubated with HepG-2 cells for 24 h. (b) Confocal microscopy imaging of HepG-2 cells and WI-38 cells treated with **M985**. Images were obtained after cells were incubated with **M985** (1 μM) for 24 h. Bottom: Flow cytometry analysis of cell staining. The data were acquired after the WI-38 cells and HepG-2 cells were incubated with **M985** (1 μM) for 24 h. Green channel: λ_{ex}= 488 nm, λ_{em}= 490−550 nm. Scale bars=20 μm

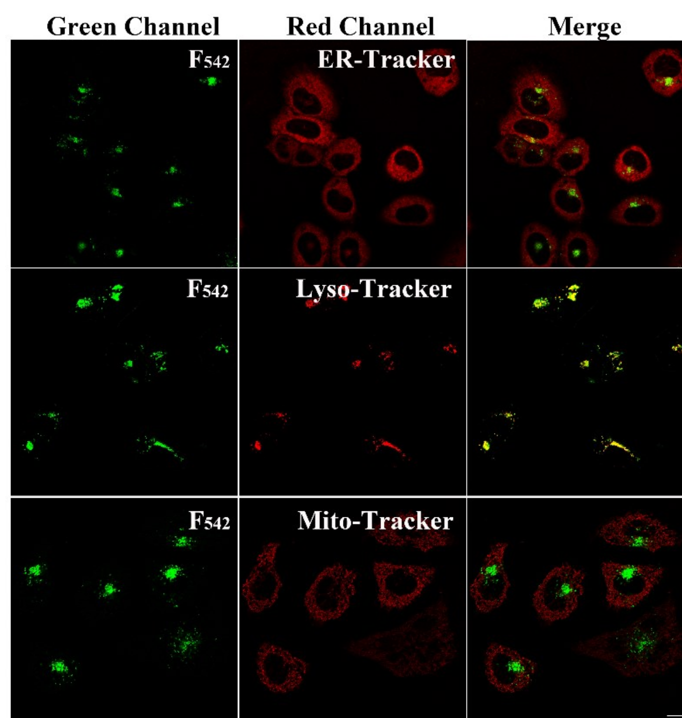


Figure S12. Confocal fluorescence images of HepG-2 cells co-incubated with F_{542} (1 μM) and ER-Tracker Red, Lyso-Tracker DND-99, Mito-Tracker Red. Images were acquired by using green channel: λ_{ex} = 488 nm, λ_{em} = 490−550 nm; red channel: λ_{ex} = 543 nm, λ_{em} = 570−650 nm. Scale bar=10 μm.

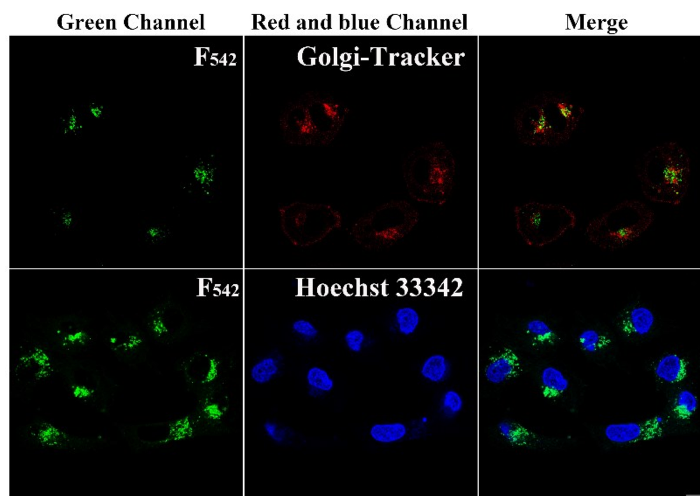


Figure S13. Confocal fluorescence images of HepG-2 cells co-incubated with F_{542} (1 μM) and Golgi-Tracker Red, Hoechst 33342. Images were acquired by using green channel: λ_{ex}= 488 nm, λ_{em}= 490−550 nm; red channel: λ_{ex}= 543 nm, λ_{em}= 570−650 nm; blue channel: λ_{ex} = 405 nm, λ_{em} = 410−460 nm. Scale bar=10 μm.

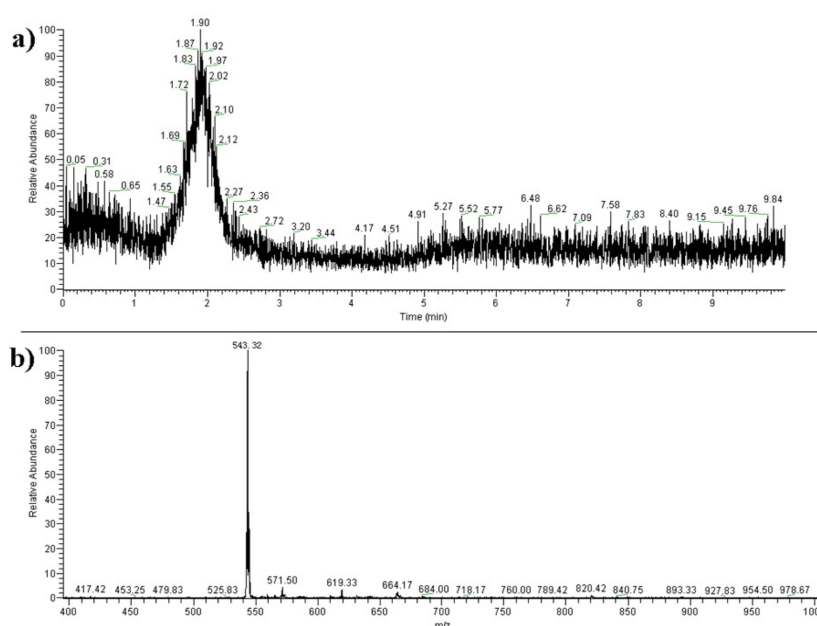


Figure S14. LC-MS analysis for the lysosomes of HepG-2 cells after the treatment of **M985** (20 μM). HPLC runs used a linear gradient from 30 % methanol/70 % H_2O to 70 % methanol/30 % H_2O over 10 min using Thermo LCQ Fleet, C8, 5μm, 2.1×150 mm column. (a) Ion flows at different time points. ESI-MS spectra of peaks eluting at (b) 1.90 min during gradient.

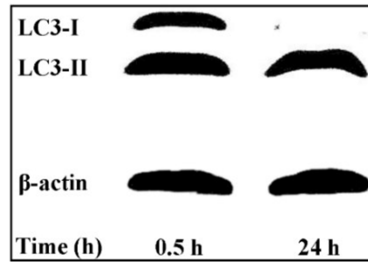


Figure S15. Western blot analysis of **M985** (5 μM) on LC3-I and LC3-II protein levels in HepG-2 cells after incubation at 0.5 h and 24 h. β-actin was used as a loading control.

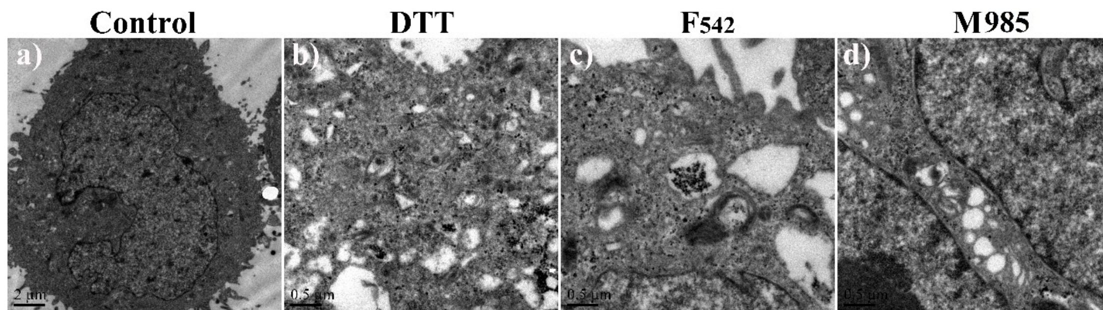


Figure S16. Transmission electron microscopy (TEM) images of HepG-2 cells. (a) Control. (b) Cells were treated with DTT (4 mM) for 12 h. (c) Cells were treated with F_{542} (10 μM) for 12 h. (d) Cells were treated with **M985** (10 μM) for 12 h.

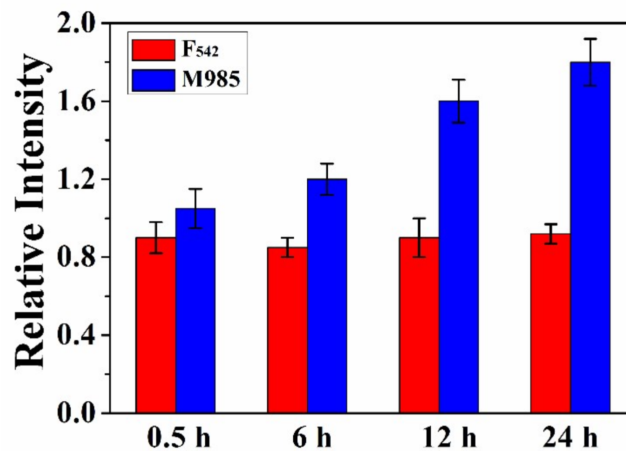


Figure S17. Time dependent analysis of intracellular ROS levels after F_{542} (5 μM) and **M985** (5 μM) treatment. HepG-2 cells were incubated at 0.5 h, 6 h, 12 h and 24 h. ROS levels are expressed as a histogram of the DHE fluorescence intensity measured by flow cytometry. The error bars represent ± S.D. (n=3).

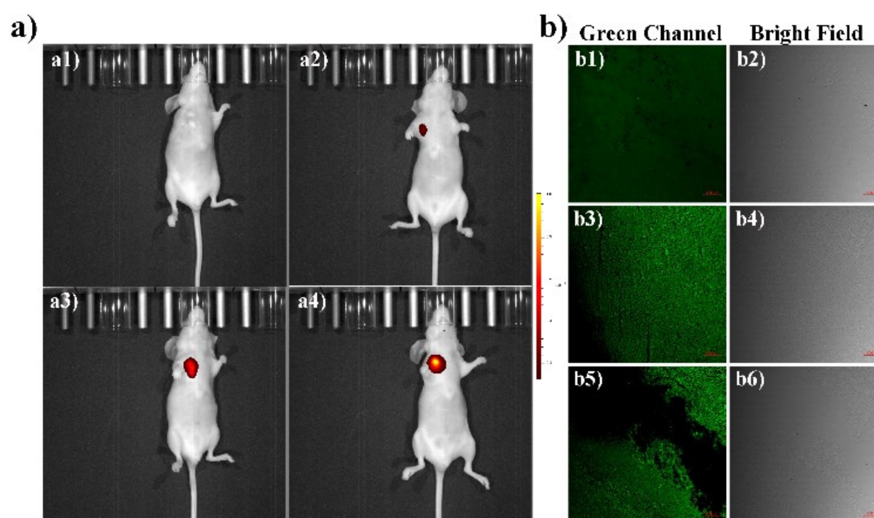


Figure. S18 (a) In vivo fluorescence imaging of **M985**-treated tumor-bearing mice at 1st day (a1), 4th day (a2), 10th day (a3) and 16th day (a4). Emission channel was collected in the range of 490–550 nm. (b) Confocal imaging of tissues from tumor-bearing mice at different stages. (b1, b2). Tissue of the tumor from a2. (b3, b4) Tissue of the tumor from a3. (b5, b6) Tissue of the tumor from a4. Green channel was collected from 490 to 550 nm. Scale bars = 100 μm.

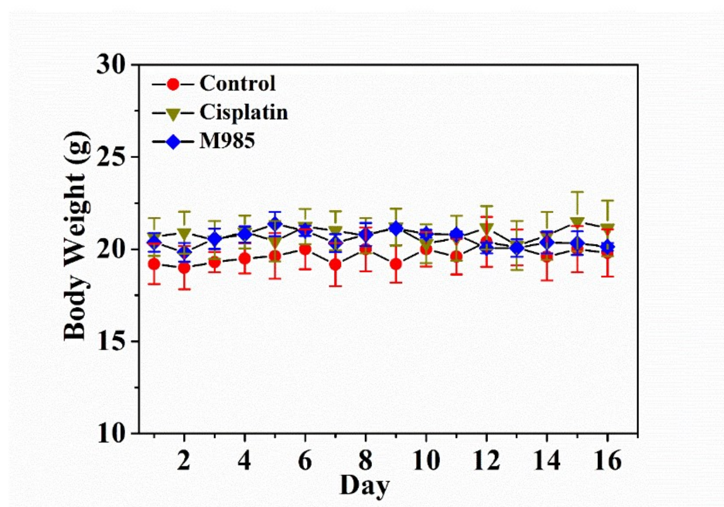


Figure S19. The body weight variation of HepG-2 tumor-bearing mice during treatment with saline, cisplatin and **M985**. The error bars represent ± S.D. (n=3).

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