Electronic Supporting Information (ESI):

Dual-target Cancer Theranostic for Glutathione S-Transferase and Hypoxia-inducible Factor-1α Inhibition

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Materials and apparatus

All of the solvents used were of analytical grade without further purification. F_{671} (5 mM) and F_{508} (10 mM) were prepared in dimethylsulfoxide (DMSO) and stored in refrigerator for use. 2, 4-Dimethyl-1H-pyrrole, 4-(chloromethyl)benzoyl chloride were purchased from Adamas-Beta. Lysosome isolation kit and NAG assay kit were purchased from ToYongBio (Shanghai, China) and Sigma-Aldrich. Dihydroethidium, Propidium Iodide and TER199 were purchased from Keygen Biotech (Nanjing, China). GSTP1-1 was expressed in *Escherichia coli* and purified as described previously.[1] GSTP1-1 activity was measured at 25 °C as reported previously.[2] NMR spectra were measured on a Bruker DRX-500 spectrometer at 25 ± 1 °C with TMS as the internal standard. Mass spectrometry data were obtained on Bruker autoflex II MALDI-TOF-MS and Thermo LCQ FLEET. Fluorescence spectra were determined on a PerkinElmer LS55 fluorescence spectrometer. Confocal fluorescence imaging was performed with a ZEISS Laser Scanning Microscope (Zeiss LSM 710). Ex vivo images were acquired by using Maestro EX in vivo imaging system. Absorption spectra were determined on a UV-3600 Shimadzu spectrometer. Lauda E100 circulating water pump was used to maintain constant temperature at 37 °C. Ultrapure water was prepared using Milli-Q A10 system. All pH measurements were made with JENCO 6230 M pH meter.

Synthesis and characterization

F_{508}, F_{465}, F_{338} were synthesized as previously reported literatures.[3]

F_{671}

F_{508} (500 mg, 0.984 mmol) and 4-Chloro-7-nitrobenzofurazan (165 mg, 0.827 mmol) were added to 30 ml chloroform, and then dropped with triethylamine (85 mg, 0.841

mmol). The resulting mixture was stirred for 8 h under N_2 at room temperature. After the solution was concentrated, the residue was re-dissolved in dichloromethane and washed three times with brine. The organic phase was dried over anhydrous Na_2SO_4. The crude product was purified by silica gel column chromatography (chloroform: methanol (10: 2 v/v)) to afford a brown solid (601 mg, 91 %). ^{1}H NMR (500 MHz, d_6-DMSO) δ H 8.55 (d, J = 9.1 Hz, 1H), 8.06 (s, 2H), 7.47 (d, J = 7.9 Hz, 2H), 7.24 (d, J = 8.0 Hz, 2H), 6.58 (d, J = 9.1 Hz, 1H), 6.19 (s, 2H), 4.24 (s, 4H), 3.81 (s, 2H), 3.18 (s, 4H), 2.69 (d, J = 6.2 Hz, 8H), 2.47 (s, 6H), 1.34 (s, 6H).^{13}C NMR (126 MHz, d_6-DMSO) δ 155.34, 143.02, 140.36, 136.44, 133.32, 131.16, 130.39, 128.13, 121.83, 104.37, 60.50, 55.01, 51.08, 47.82, 46.96, 14.61. MALDI-TOF-MS: Calcd. F_{671} $[M+H]^+$: 672.339, found 672.339.

F_{335}

1, 4, 7, 10-tetraazacyclododecane (1.75 g, 10.174 mmol) and 4-Chloro-7-nitrobenzofurazan (200 mg, 1.002 mmol) were added to 60 ml chloroform, and dropped with triethylamine (100 mg, 0.990 mmol). The resulting mixture was stirred for 12 h under N_2 at room temperature. After the solution was concentrated, the residue was re-dissolved in dichloromethane and washed three times with brine. The organic phase was dried over anhydrous Mg_2SO_4. The crude product was purified by silica gel column chromatography (chloroform: methanol: ammonium hydroxide (10: 1: 0.1 v/v/v)) to afford a deep red solid (300 mg, 89 %). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.47 (d, J = 9.1 Hz, 1H), 6.59 (d, J = 9.2 Hz, 1H), 4.19 (s, 4H), 3.38 (s, 3H), 3.05 (s, 3H), 2.63 (d, J = 10.0 Hz, 8H). ^{13}C NMR (101 MHz, d^6-DMSO) δ 146.35, 144.84, 120.52, 103.66, 55.07, 48.22, 46.72, 45.55. MALDI-TOF-MS: Calcd. F_{335} $[M+H]^+$: 336.178, found 336.178.

Determination of quantum yields[4]

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

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

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

Cell lines and culture conditions

HepG-2, HeLa, A549, MCF-7, LO2 cells were maintained following protocols provided by the American Type Tissue Culture Collection. Cells were maintained in DMEM (Dulbecco's modified Eagle's medium, Gibco BRL) or 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 cells were cultured in a humidified incubator, which provided an atmosphere of 5 % CO_2 and 95 % air at a constant temperature of 37 °C. The cisplatin resistant clone (A549cisR) of A549 cells were established by exposing the cells with gradually increasing concentrations of cisplatin until chemoresistance acquisition was elucidated by MTT and A549cisR cells could stable grow and be passaged.

Cell viability assay

The cytotoxicity of the tested compounds towards different cell lines was determined by MTT assay. Cells were seeded in a 96-well plate at 5×10^3 cells per well and allowed to grow 24 h prior to exposure to different amounts of compounds for further incubation time. 20 μL of MTT solution (5 mg/mL) was then added and the cells were incubated for another 4 h, DMSO (150 μL/well) was further incubated with cells for 10 min after removing the medium. The absorbance at 490 nm was recorded in a Varioskan Flash microplate reader. The following formula was used to calculate the cell viability: Cell viability (%) = (mean of A value of treatment group/mean of A value of control) × 100.

Transmission electron microscopy

HepG-2 cells were treated with F_{671} (10 μM), F_{508} (10 μM), Cisplatin (50 μM) at 37 ºC for 12 h. Cells were collected and fixed overnight at 4 ºC in phosphate buffer (pH 7.4) containing 2.5 % glutaraldehyde. Subsequently, the cells were treated with

osmium tetroxide, stained with uranyl acetate and lead citrate, and visualized using a transmission electron microscope (JEM 100 CX, JEOL, Tokyo, Japan). Images were photographed by the Eversmart Jazz program (Scitex).

Western blot analysis

HepG-2 cells were seeded into 6-well plates and incubated for 24 h under 21 % O_2, and then exposed to varied concentrations of F_{671}, F_{508} for 24 h under 1 % O_2. Cells were harvested and washed with ice cold PBS twice. 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% nonfat 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 (1: 4000) antibody and an anti-mouse secondary (1: 4000) antibody for 1 h at room temperature, respectively. Enhanced chemiluminescence (ECL, Millipore) was performed afterwards.

RNA isolation and reverse transcription PCR

Total RNA was extracted from cultured cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The RNA concentration was quantified to two micrograms of each sample through Nanodrop ND-1000 (Thermo Scientific, USA). Each RNA sample was reversely transcribed into cDNA by PrimeScript reverse transcriptase using a PrimeScript RT-PCR kit. PCR proceeded using the cDNA as a template and TakaRa Taq TM kit by following the manufacturer's instructions. The number of PCR cycles determined from the plot was 30 for HIF-1α, and 25 for β-actin. The amount of amplified product was detected by 0.1 % agarose gel electrophoresis, scanned and analyzed using Quantity One (Bio-Rad, Hercules, CA, USA). Each sample was assayed in triplicate.

Co-localization assay

HepG-2 cells were incubated with F_{508} (1 μM) at 37 °C for 0.5 h and further co-incubated with Mito Tracker Red CMXRos (100 nM) or Lyso Tracker DND-99 (100 nM) at 37 °C for 1 h. Cells were washed three times with ice-cold PBS and visualized by confocal microscopy (LSM 710, Carl Zeiss). Images were acquired by using green channel: λ_{ex}= 488 nm, λ_{em}= 490-550 nm; Red channel: λ_{ex}= 543 nm, λ_{em}= 570-630 nm.

Flow Cytometry

HepG-2 cells were plated into flat-bottomed culture dishes (Φ 60 mm) containing 5 mL of DMEM. After incubation at 37 °C with 5 % CO_2 for 2 days, the media was replaced with fresh DMEM, and F_{671} (F_{508}) was added for different time spans. The control group was treated with F_{671} (F_{508}) for 20 min. PI or DHE was stained for 0.5 h at 37 °C before FCM analysis. Samples were illuminated with a sapphire laser at 488 nm on a BD LSRFortessa flowcytometer. The fluorescence of the forward-scattered and side-scattered light from 10000 cells were detected at rate of 150 events/s. Flow cytometry data were analyzed with FlowJo software.

Lysosome Isolation and LC-MS Analysis

HepG-2 cells were plated into flat-bottomed culture dishes (Φ 100 mm) containing 10 mL of DMEM. After incubation at 37 °C with 5 % CO_2 for 1 d, the media was replaced with fresh DMEM, and 10 μM F_{671} were added and incubated for 24 h. The cells were harvested and washed with ice-cold PBS twice. Then the cells were re-suspended with ice-cold PBS, counted and centrifuged for 5 min at 600 g under 4 °C, and the supernatant was discarded. The packed cell volume should be 1.5-3 ml. The next procedures were strictly followed as the technical bulletin of Lysosome Isolation Kit from Sigma-Aldrich. The isolated lysosomes of HepG-2 were subsequently crushed in ice bath using ultrasonic to afford clear solution. Methanol was added and the solution was centrifuged for 20 min at 5000 g under room temperature, the supernatant was collected for LC-MS analysis. 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.

Lysosome Membranes Integrity Assay

The integrity of the lysosome membrane was monitored by measuring the released β-N-acetylglucosaminidase (NAG) activity. Lysosomes were isolated from HepG-2 cells treated with F_{671} (1 μM) and F_{508} (1 μM). The released NAG and total NAG of each sample were determined with Lysosomal Membrane Integrity Kit. The reaction mixture was incubated for 5 min at 37 °C and then stopped using 10 μL stop solution. The fluorescence was measured using Varioskan Flash microplate reader (Ex 365 nm, Em 444 nm). The integrity of lysosome membrane was represented by the percentage of released NAG/total NAG.

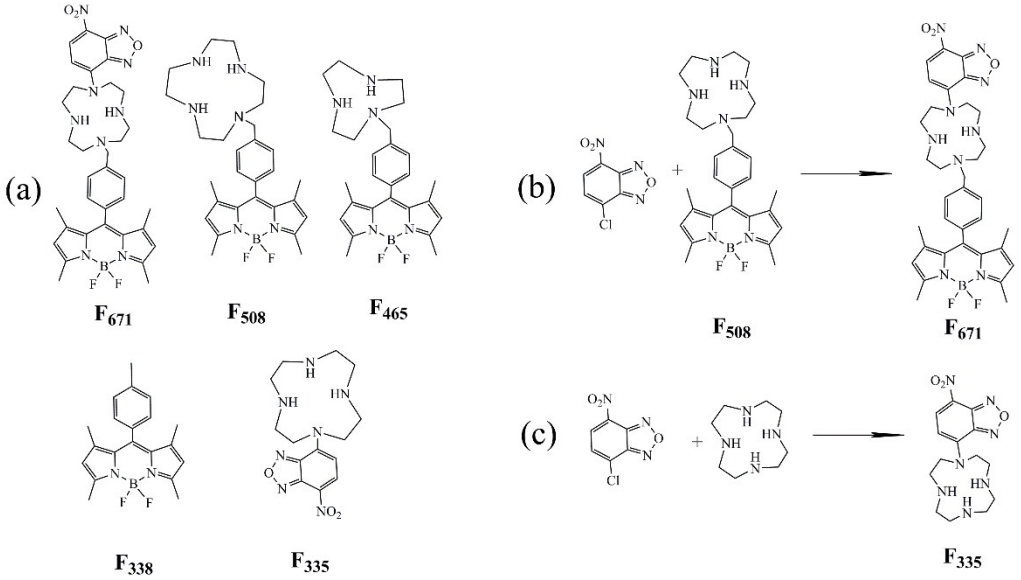
Animal and Tumor Model

Specific pathogen-free (SPF) female BALB/c mice, four weeks of age, were purchased from Shanghai Laboratory Animal Center 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 model was established by subcutaneous injection of HepG-2 cells (1×10^6) into the selected positions of the nude mice. Each tumor's volume was calculated using the following formula: tumor volume = length × width^2 × 0.5.

In vivo antitumor efficacy

The tumor-bearing mice were weighed and randomly divided into four groups when the tumor volume reached to 30 mm^3, and subjected to the following treatments: 1. Saline; 2. 5 mg/Kg body weight; 3. 10 mg/Kg body weight; 4. 50 mg/Kg body weight. The mice were injected by intragastric administration, successive medication for 16 d, and meanwhile the tumor sizes and body weights were measured. At Day 17th, the mice were euthanized, and the tumors were collected, weighed, washed with saline thrice and fixed in the 10 % neutral-buffered formalin.

Supporting Tables and Figures



Scheme S1 (a) The structure of molecules in use. (b) Synthesis of F_{671}. (c) Synthesis of F_{335}.

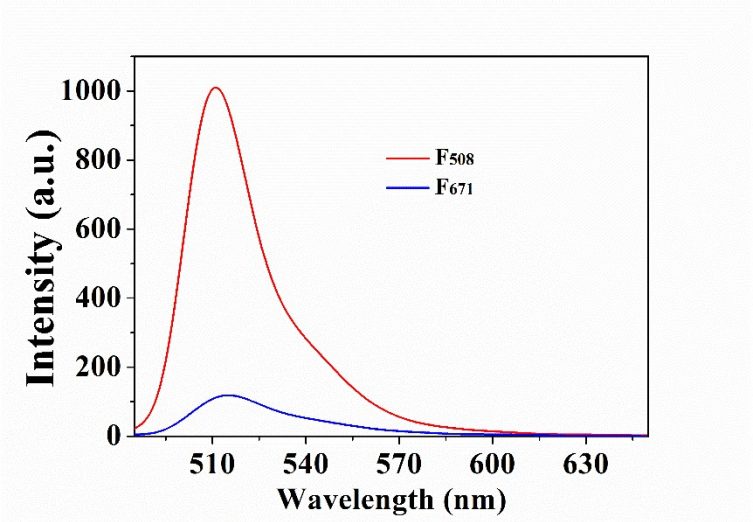


Figure S1. The fluorescence spectra of F_{671} (10 μM) (blue) and F_{508} (10 μM) (red) in Tris-HCl (0.02 M) buffer (DMSO/Tris-HCl = 1:9 v/v, pH 7.4) at 37 °C. Slit width 2.5 nm, excitation wavelength 480 nm.

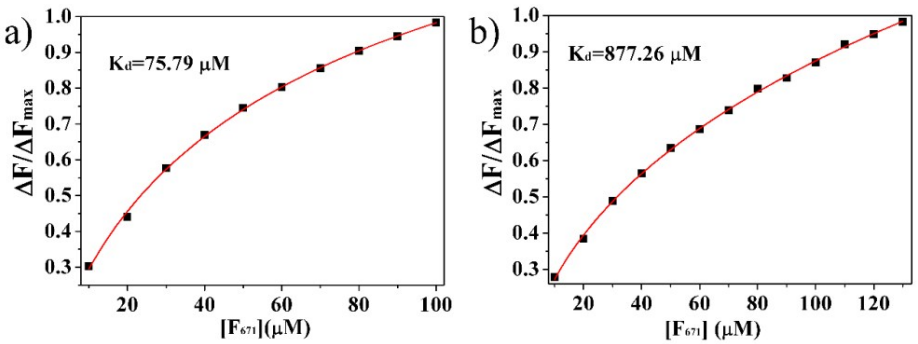


Figure S2. Isothermic binding of F_{671} to GSTP1-1. The binding of F_{671} was studied at 25 °C by following the quenching of the intrinsic fluorescence of the protein. Binding of F_{671} to GSTP1-1 in the presence of 1 mM GSH (a) and in the absence of GSH (b).

In vitro cytotoxicity

Table S1. Cytotoxic effects of different compounds toward several human cell lines for 48 h. Cell viability was assayed with MTT test. The error represent ± S.D. (n= 3).

	IC_{50} (μM)					
Compound	**HepG-2**	**Hela**	**A549**	**A549cisR**	**MCF-7**	**LO2**
F_{671}	4.73±0.31	6.73±0.47	6.89±0.33	5.31±0.51	5.35±0.36	7.13±0.57
F_{508}	10.07±0.91	8.11±0.64	8.71±0.73	9.10±0.95	6.79±0.78	10.83±1.31
Cisplatin	7.65±0.80	9.38±0.53	10.18±0.97	21.04±1.66	12.40±1.05	6.42±0.25
F_{465}	16.33±1.61	24.35±1.42	20.12±2.05	25.12±3.23	28.33±2.82	31.82±3.13
F_{335}	21.23±1.62	28.23±1.89	32.56±3.01	26.23±1.53	18.15±1.24	30.53±3.17
F_{338}	59.54±4.75	>100	>100	60.54±5.21	71.75±5.97	>100

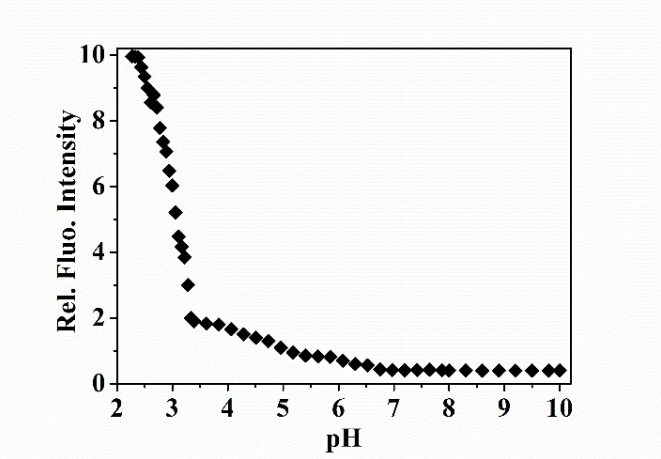
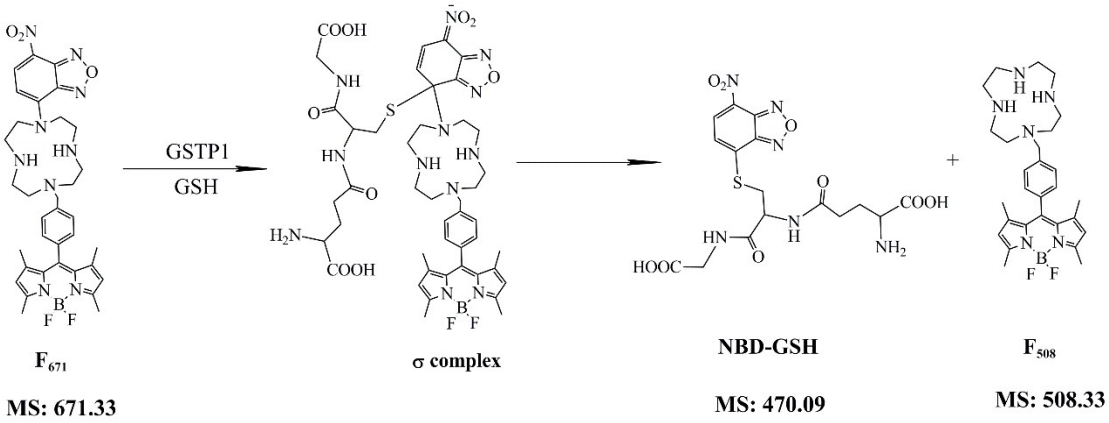


Figure S3. Relative fluorescence intensity of F_{671} (10 μM) at 510 nm as a function of pH. Slit width was 2.5 nm, excitation wavelength was 480 nm. All measurements were taken at 37 °C.

Proposed responding mechanism



Scheme S2. Proposed reaction mechanism of F_{671} with GSTP1 in Tris-HCl buffer solution.

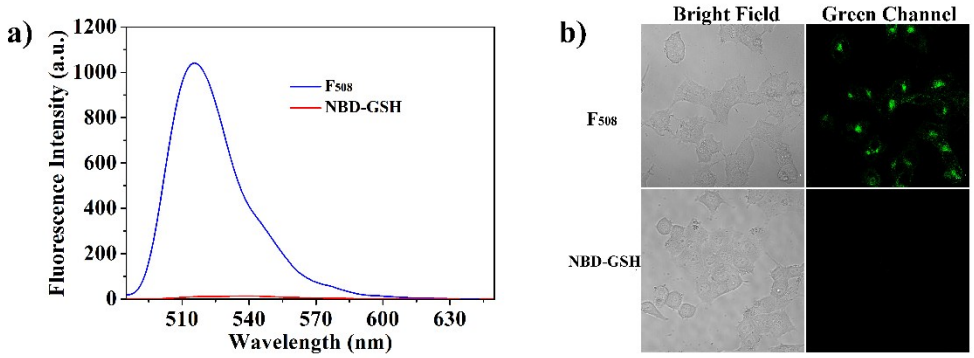


Figure S4. (a) The fluorescence spectra of F_{508} (1 μM) and NBD-GSH (1 μM) in Tris-HCl (0.02 M) buffer (DMSO/Tris-HCl = 1:9 v/v, pH 7.4) at 37 °C. Slit width 10 nm, excitation wavelength 480 nm; (b) Confocal fluorescence images of HepG-2 cells co-incubated with F_{508} (1 μM) and NBD-GSH (1 μM) for 1 hour . Images were acquired by using green channel: λ_{ex} = 488 nm, λ_{em} = 490-550 nm; red channel: λ_{ex} = 543 nm, λ_{em} = 570-630 nm.

ESI-MS spectra

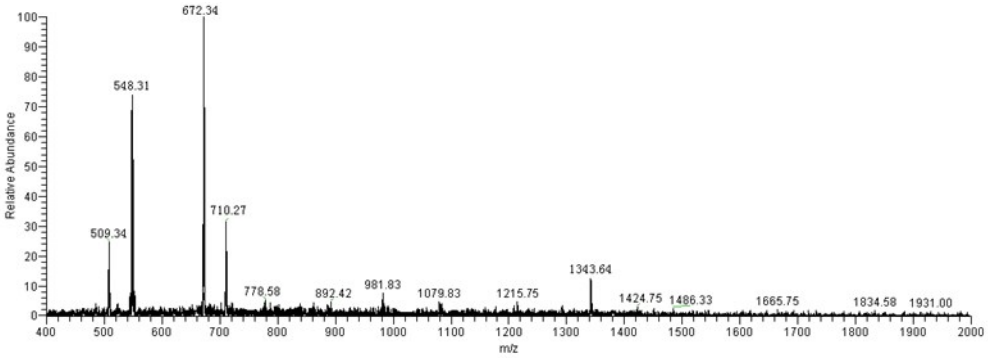


Figure S5. Positive ESI-MS spectrum of F_{671} in the presence of GSTP1-1 and GSH.

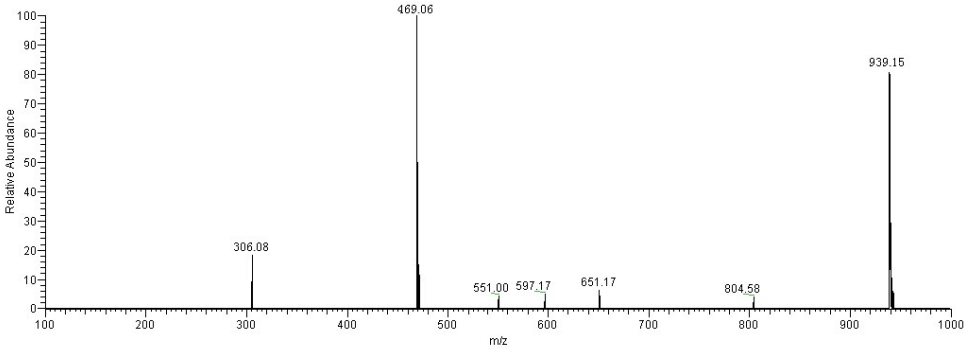


Figure S6. Negative ESI-MS spectrum of F_{671} in the presence of GSTP1-1 and GSH.

Confocal fluorescence images

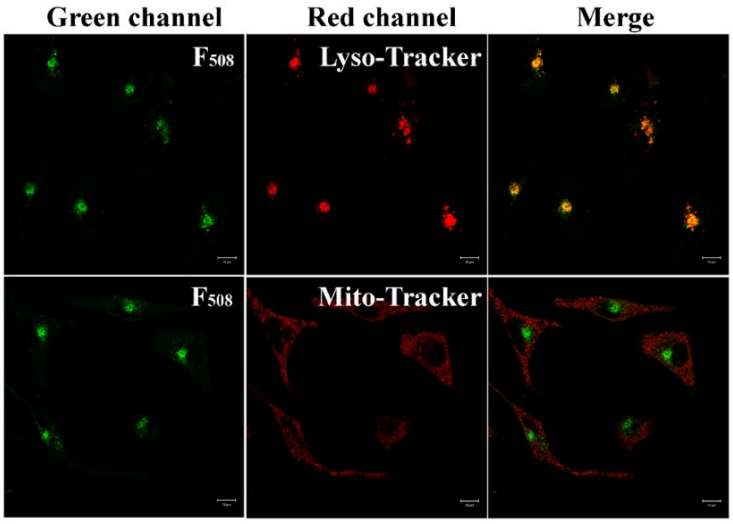


Figure S7. Confocal fluorescence images of HepG-2 cells co-incubated with F_{508} (1 μM) and Lyso-Tracker DND-99, Mito-Tracker Red. Images were acquired by using

green channel: λ_{ex} = 488 nm, λ_{em} = 490-550 nm; red channel: λ_{ex} = 543 nm, λ_{em} = 570-630 nm.

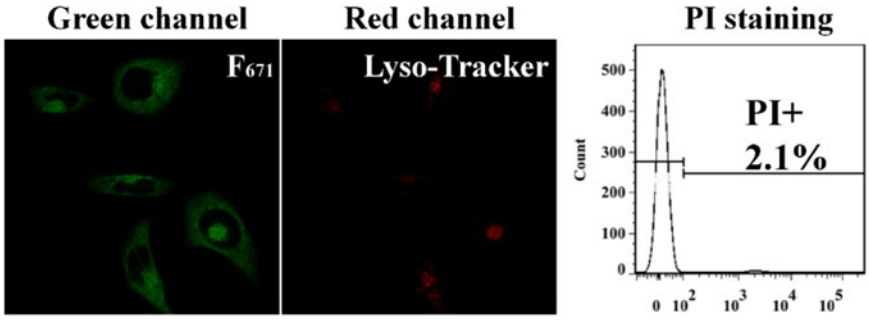


Figure S8. Confocal fluorescence images of HepG-2 at 0.5 h after incubating with F_{671} (1 μM) for 20 min. Flow cytometry analysis of PI positive cells at 0.5 h. Images were acquired using green channel: λ_{ex}= 488 nm, λ_{em}= 490-550 nm; and red channel: λ_{ex}= 543 nm, λ_{em}= 570-630 nm.

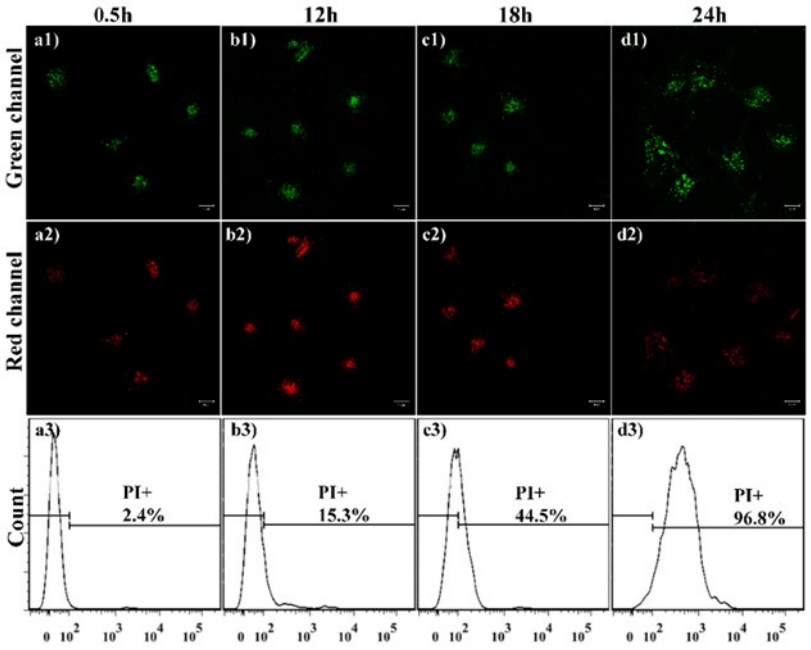


Figure S9. Lysosome morphological variation in HepG-2 cells after different incubation time with F_{508} (1 μM) treatment. (a1, b1, c1, d1) The green fluorescence distribution at 0.5 h,12 h, 18 h, and 24 h; (a2, b2, c2, d2) Lyso-Tracker DND-99 staining exhibits the morphology alteration of lysosomes at 0.5 h, 12 h, 18 h, and 24 h; (a3, b3, c3, d3) Flow cytometry analysis of PI positive cells at 0.5 h, 12 h, 18 h, and 24 h. Images were acquired using green channel: λ_{ex}= 488 nm, λ_{em}= 490-550 nm; and red channel: λ_{ex}= 543 nm, λ_{em}= 570-630 nm.

NAG activity assay

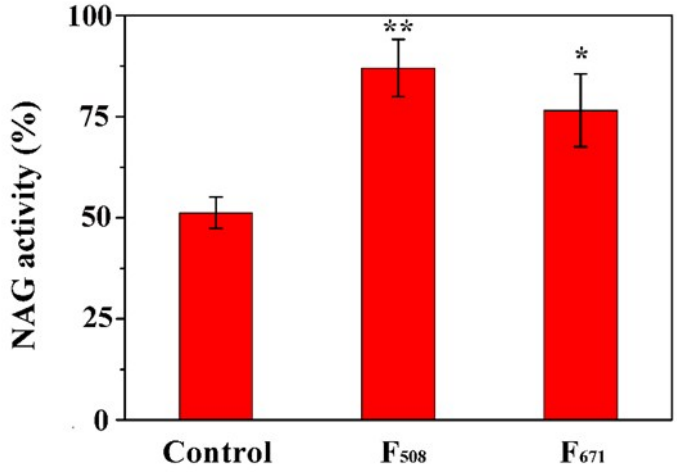


Figure S10 The released NAG activity. HepG-2 cells were incubated with F_{508} (1 μM) and F_{671} (1 μM) for 24 h. The NAG activity was measured after the isolation of lysosomes from three group HepG-2 cells (Control, F_{508}-treated, F_{671}-treated). The error bars represent ± S.D. (n=3), *P < 0.05, **P < 0.01.

LC-MS spectra

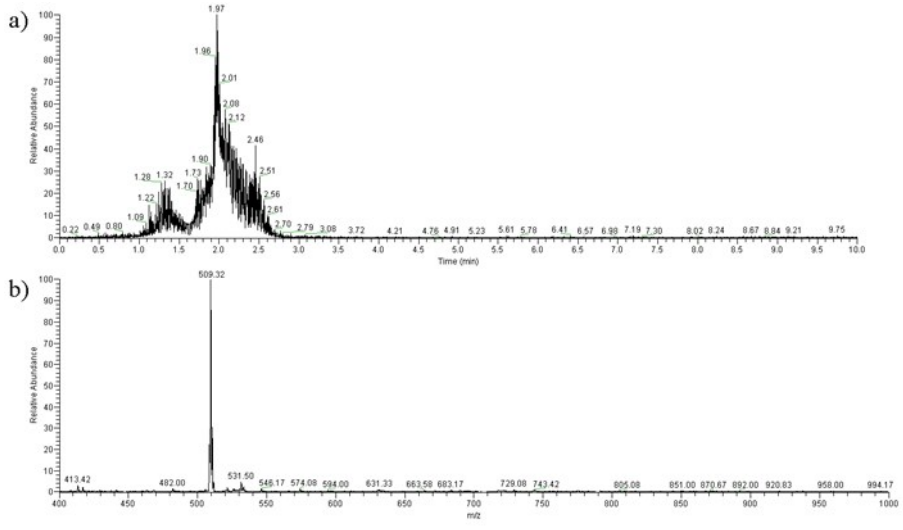


Figure S11. LC-MS analysis for the lysosomes of HepG-2 cells after F_{671} treatment. HPLC runs used a linear gradient from 40 % methanol / 60 % H_2O to 80 % methanol/ 20 % H_2O over 10 min using Thermo LCQ Fleet, C8, 5μm, 2.1×150 mm column. (a)

Ion flows at different time points. Positive mass spectra of peaks eluting at (b) 1.32 min during gradient.

TEM assay

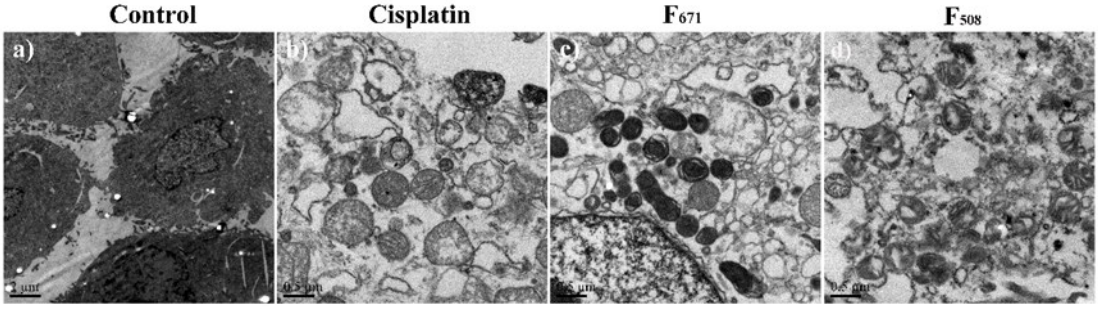


Figure S12. TEM images of HepG-2 cells. (a) Control. (b) Cells were treated with cisplatin (10 μM) for 12 h. (c) Cells were treated with F_{671} (10 μM) for 12 h. (d) Cells were treated with F_{508} (10 μM) for 12 h.

Western blot assay

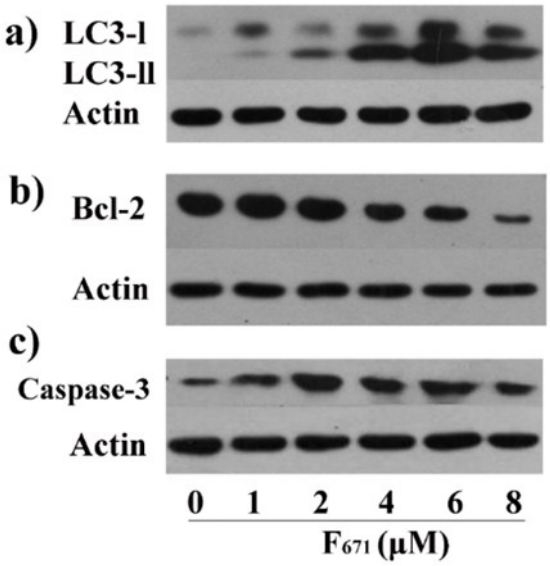


Figure S13. Effects of F_{671} treatment on protein levels of LC3, Bcl-2 and active-caspase-3 in HepG-2 cells detected by western blot. HepG-2 cells were incubated with varied concentrations (0 μM, 1 μM, 2 μM, 4 μM, 6 μM, 8 μM) of F_{671} for 24 h. (a) The relative protein levels of LC3 II / LC3 I upon various concentrations of F_{671}.

(b) The protein levels of Bcl-2 upon varied concentrations of F_{671}. (c) The protein levels of activated caspase-3 (17 kDa) upon various concentrations of F_{671}.

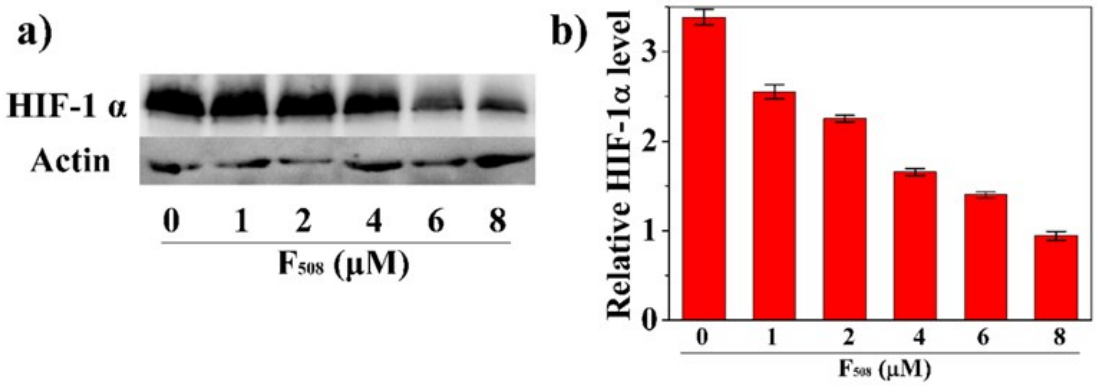


Figure S14. Effects of F_{508} treatment on protein levels of HIF-1α in HepG-2 cells under hypoxia (1 % O_2). HepG-2 cells were incubated with various concentrations (0 μM, 1 μM, 2 μM, 4 μM, 6μM, 8 μM) of F_{508} for 24 h. (a) HIF-1α accumulation was assessed by western blot analysis in whole cell extracts. β-actin was used as a loading control. (b) Quantification of HIF-1α protein expression levels by densitometry relative to β-actin. The error bars represent ± S.D. (n=3)

ROS levels

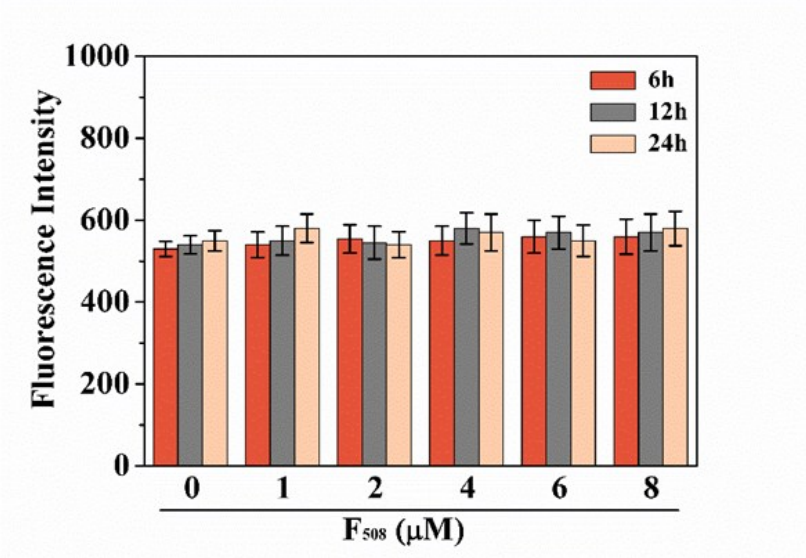


Figure S15. Time- and concentration-dependent analysis of intracellular ROS levels caused by F_{508} treatment. HepG-2 cells were incubated with various concentrations of F_{508} for 6 h, 12 h, 24 h. ROS levels are expressed as a histogram of the DHE

fluorescence intensity measured by flow cytometry. The error bars represent ± S.D. (n=3).

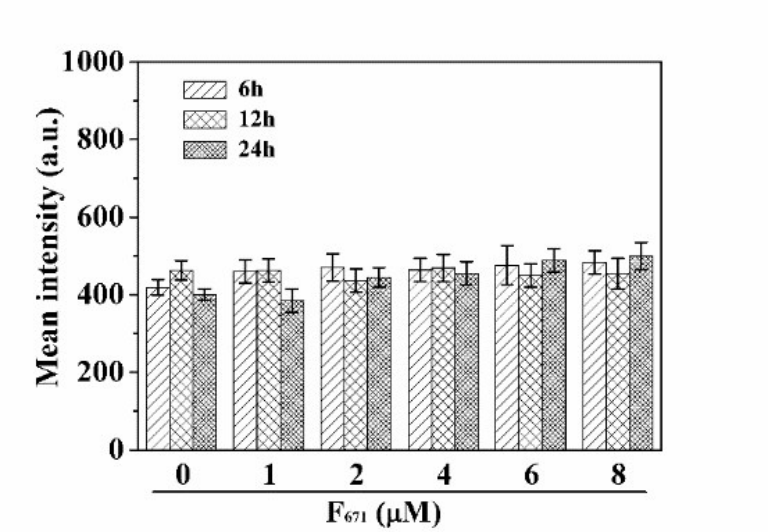


Figure S16. Time- and concentration-dependent analysis of intracellular ROS levels caused by F_{671} treatment. The HepG-2 cells were incubated with various concentrations of F_{671} for 6 h, 12 h and 24 h. ROS levels are expressed as a histogram of the DHE fluorescence intensity measured by flow cytometry. The error bars represent ± S.D. (n=3).

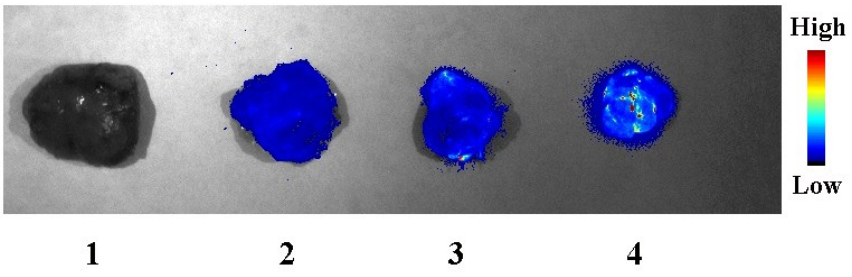


Figure S17. Representative fluorescence images of the HepG-2 tumors after dealing with different treatments at day 16 (1: saline, 2: low-dose, 3: medium-dose, 4: high-dose).

References

[1] M. Lo Bello, A. Battistoni, A. P. Mazzetti, P. G. Board, M. Muramatsu, G. Federici, G. Ricci, J. Biol. Chem. **1995**, 270, 1249-1253.

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