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Electronic Supplementary Information (ESI)

A vinyl-decorated covalent organic framework for ferroptotic cancer therapy *via* visible-light-triggered cysteine depletion

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Supplemental figures

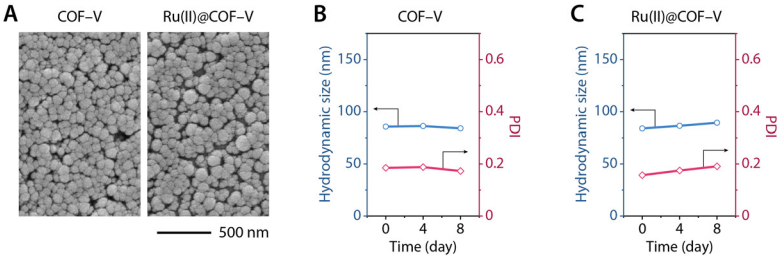

Figure S1. Additional characterization data of COF-V and Ru(II)@COF-V.

(A) SEM images of COF-V and Ru(II)@COF-V.

(B–C) Time-dependent z-average particle size and PDI values of COF-V (B) and Ru(II)@COF-V (C) in PBS.

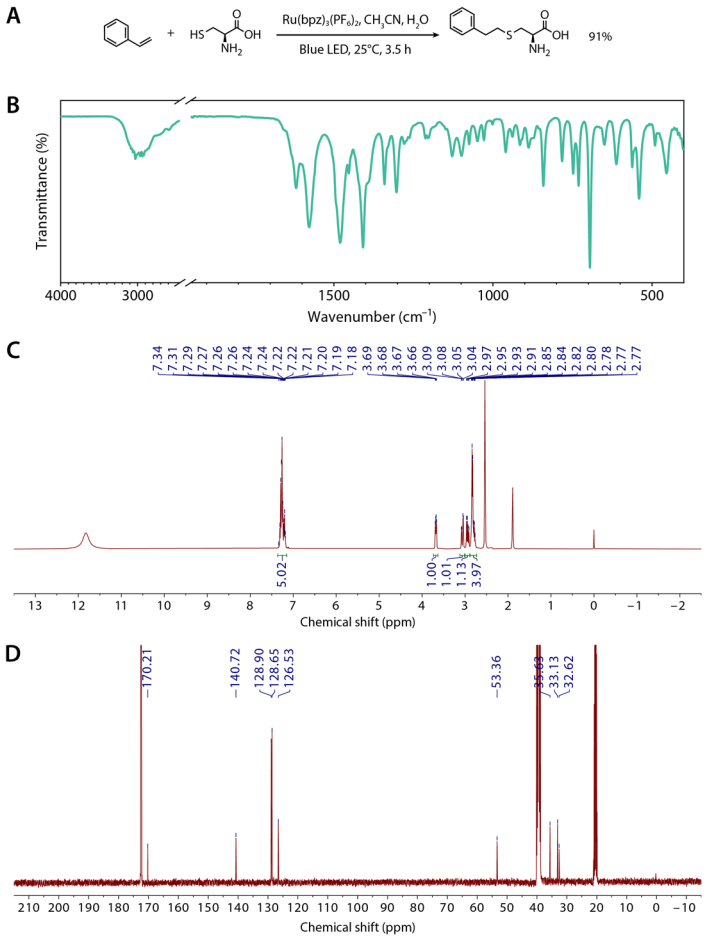

Figure S2. Synthesis and characterization of *S*-phenethyl-*L*-cysteine.

(A) Synthesis of *S*-phenethyl-*L*-cysteine by the thiol–ene reaction of *L*-cysteine with styrene.

(B) ATR–FTIR spectrum of *S*-phenethyl-*L*-cysteine.

(C) ^{1}H NMR spectrum of *S*-phenethyl-*L*-cysteine in DMSO-d_6/CD_3COOD (5:1, v/v).

(D) ^{13}C NMR spectrum of *S*-phenethyl-*L*-cysteine in DMSO-d_6/CD_3COOD (5:1, v/v).

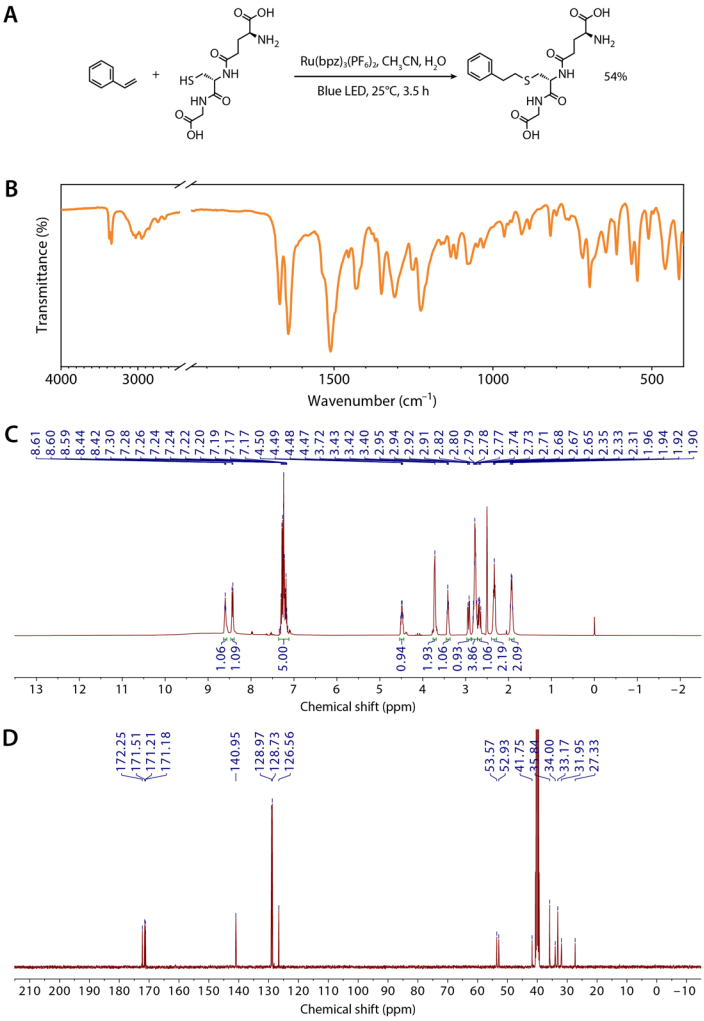

Figure S3. Synthesis and characterization of *S*-phenethyl-*L*-glutathione.

(A) Synthesis of *S*-phenethyl-*L*-glutathione by the thiol–ene reaction of GSH with styrene.

(B) ATR–FTIR spectrum of *S*-phenethyl-*L*-glutathione.

(C) ^{1}H NMR spectrum of *S*-phenethyl-*L*-glutathione in DMSO-d_6.

(D) ^{13}C NMR spectrum of *S*-phenethyl-*L*-glutathione in DMSO-d_6.

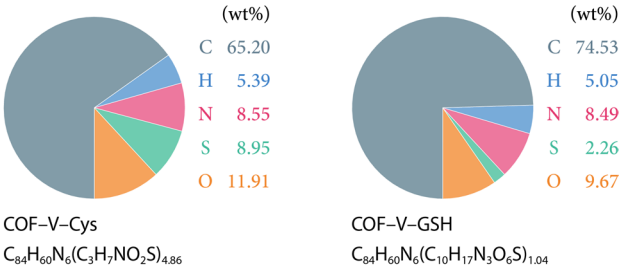

Figure S4. Elemental analysis data of COF-V-Cys and COF-V-GSH. The oxygen content was calculated from the measured values of other elements.

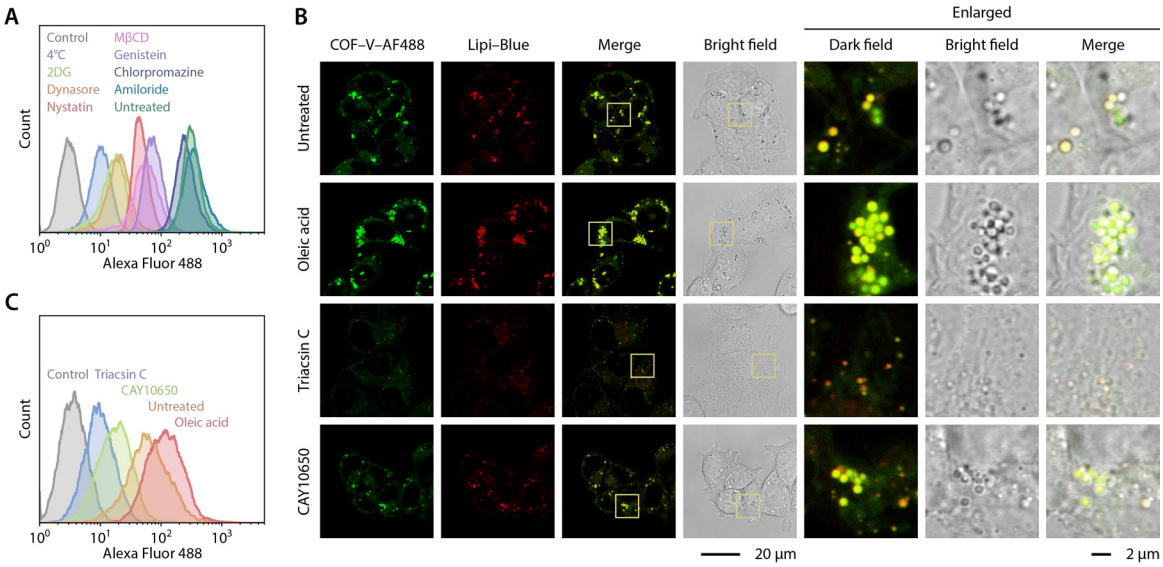

Figure S5. Cellular uptake of COF-V.

(A) Flow cytometry analysis of energy-dependent pinocytosis of COF-V-AF488 (200 μg/mL, 2 h) toward HCT-116 cells pretreated with 2DG (150 mM), dynasore (75 μM), nystatin (25 μM), MβCD (10 mg/mL), genistein (40 μM), chlorpromazine (50 μM), and amiloride (0.2 mM) for 1 h.

(B) Colocalization of COF-V-AF488 (100 μg/mL, 2 h) and lipid droplets labeled with Lipi-Blue (0.1 μM, 15 min) toward HCT-116 cells pretreated with oleic acid (200 μM, 12 h), triacsin C (5.0 μM, 12 h), and CAY10650 (20 nM, 1 h).

(C) Flow cytometry analysis of lipid droplet content-dependent cellular uptake of COF-V-AF488 (100 μg/mL, 2 h) toward HCT-116 cells preincubated with oleic acid (200 μM, 12 h), triacsin C (2.0 μM, 12 h), and CAY10650 (20 nM, 1 h).

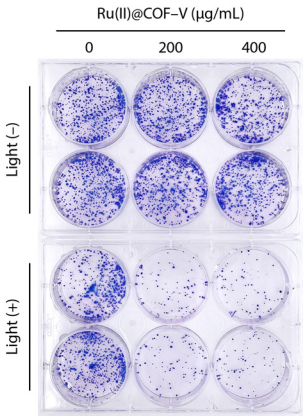

Figure S6. *In vitro* clonogenic assays of HCT-116 cells treated with Ru(II)@COF-V (0–400 μg/mL) under a blue LED light (0 or 20 mW/cm^2, 15 min).

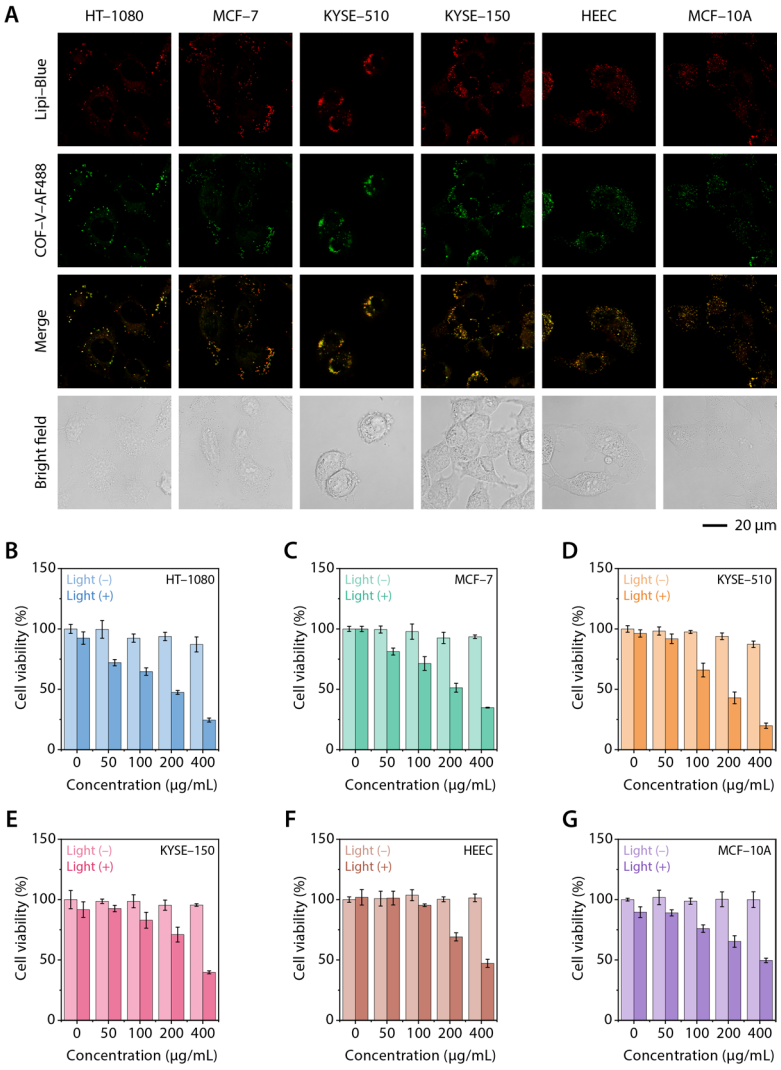

Figure S7. *In vitro* antitumor experiments of HT-1080, MCF-7, KYSE-510, KYSE-150, HEEC, and MCF-10A cells.

(A) Colocalization of COF-V-AF488 (100 μg/mL, 2 h) and lipid droplets labeled with Lipi-Blue (0.1 μM, 15 or 30 min).

(B–G) CCK-8 cell viability assays of HT-1080 (B), MCF-7 (C), KYSE-510 (D), KYSE-150 (E), HEEC (F), and MCF-10A (G) cells that were treated with Ru(II)@COF-V (0–400 μg/mL, 2 h), exposed to a blue LED light (0 or 20 mW/cm^2, 15 min), and cultured for 24 h. Data are presented as the mean ± SD (n = 3).

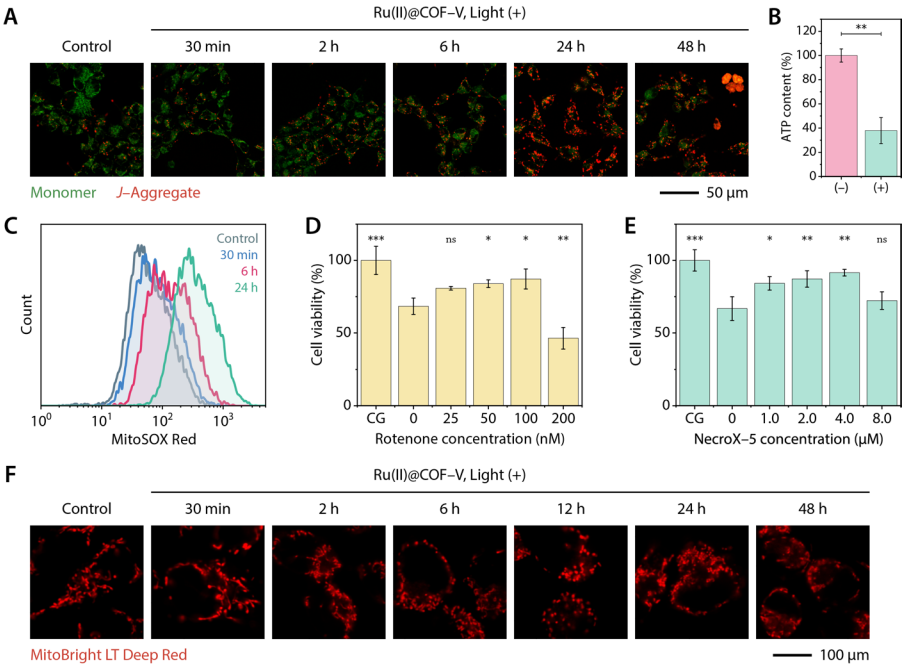

Figure S8. Mitochondria-associated cell damage.

(A) Time-dependent mitochondrial membrane potentials of HCT-116 cells that were treated with Ru(II)@COF-V (0 or 100 μg/mL, 2 h), exposed to a blue LED light (0 or 20 mW/cm^2, 15 min), and cultured for 0.5–24 h.

(B) Intracellular ATP contents of HCT-116 cells that were treated with Ru(II)@COF-V (200 μg/mL, 2 h), exposed to a blue LED light (0 or 20 mW/cm^2, 15 min), and cultured for 12 h.

(C) Mitochondrial superoxide levels of HCT-116 cells that were treated with Ru(II)@COF-V (0 or 100 μg/mL, 2 h), exposed to a blue LED light (0 or 20 mW/cm^2, 15 min), and cultured for 24 h.

(D–E) Cell viability of HT-116 cells treated with Ru(II)@COF-V (100 μg/mL, 2 h) under a blue LED light (0 or 20 mW/cm^2, 15 min) and cultured for 24 h in the presence of rotenone (D) or NecroX-5 (E).

(F) Morphology of mitochondrial networks of HCT-116 cells that were treated with Ru(II)@COF-V (0 or 100 μg/mL, 2 h), exposed to a blue LED light (0 or 20 mW/cm^2, 15 min), and cultured for 0.5–48 h.

Data are presented as the mean ± SD (n = 3) and compared by Welch's t test (B) or one-way ANOVA followed by Dunnett's multiple comparison test (D and E). ***p < 0.001; **p < 0.01; *p < 0.05; *ns*, no significance (p > 0.05).

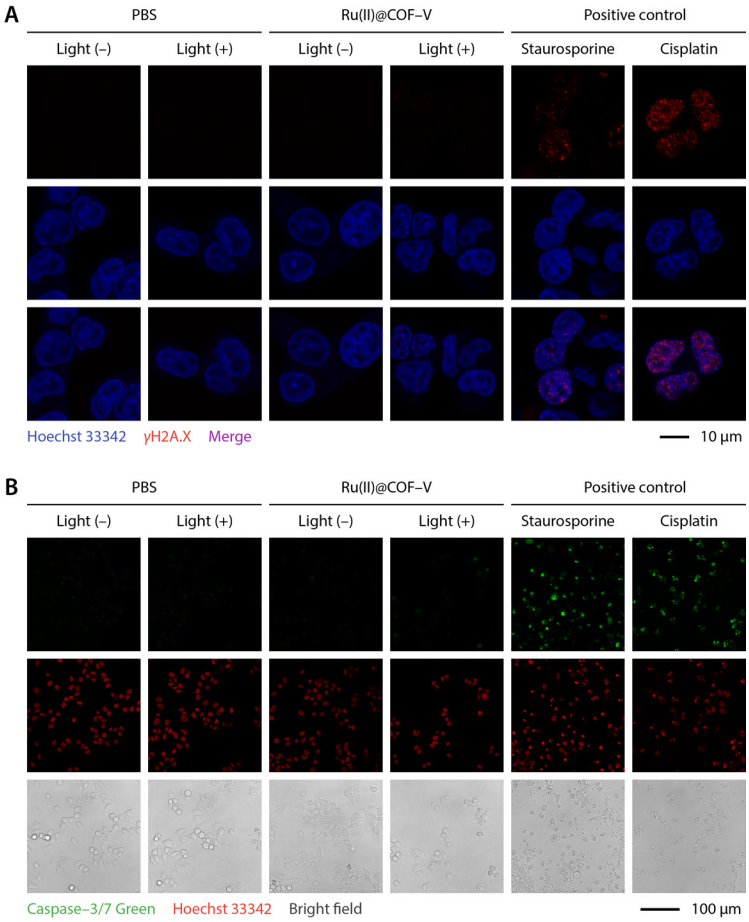

Figure S9. Apoptosis-independent cell death of HCT-116 cells treated with Ru(II)@COF-V (0 or 200 μg/mL, 2 h) and exposed to a blue LED light (0 or 20 mW/cm^2, 15 min).

(A) γH2A.X immunofluorescence staining.

(B) Caspase 3 activation assays.

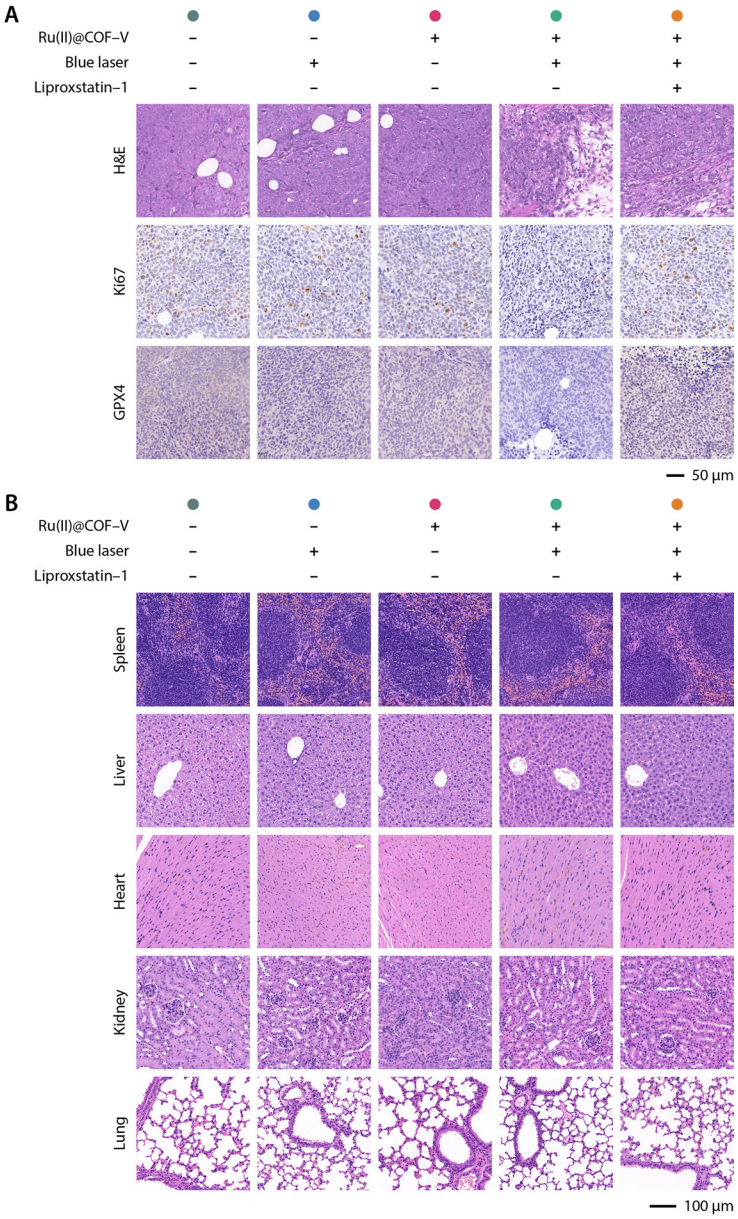

Figure S10. Histological analysis.

(A) H&E staining, Ki67 immunohistochemical staining, and GPX4 immunohistochemical staining of the obtained tumors.

(B) H&E staining of the major organs (spleen, liver, lung, heart, and kidney) obtained by dissection at day 12.

Supplemental experimental procedures

Experimental materials

Methyl acetate, *n*-butanol, styrene, glutathione (GSH), and oxidized glutathione (GSSG) were purchased from Macklin (Shanghai, China).

L-cysteine hydrochloride monohydrate, cisplatin, belnacasan, and triacsin C were purchased from Aladdin (Shanghai, China).

Methanol, ethanol, acetic acid, and acetonitrile were purchased from Sinopharm (Beijing, China).

$Ru(bpz)_3(PF_6)_2$ (bpz = 2,2'-bipyrazine) was purchased from Leyan (Shanghai, China).

1,3,5-Tris(4-aminophenyl)benzene was purchased from Kylpharm (Shanghai, China).

2,5-Divinylterephthalaldehyde was purchased from Jilin Chinese Academy of Sciences – Yanshen Technology (Changchun, China).

Glutathione ethyl ester (GSH-OEt) and poly(2-hydroxyethyl methacrylate) were purchased from Sigma–Aldrich (Shanghai, China).

Tween-20, Triton X-100, oleic acid, 3-methyladenine, 2-mercaptoethanol, *N*-acetylcysteine, and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were purchased from TCI (Shanghai, China).

Hoechst 33342 trihydrochloride, propidium iodide, staurosporine, α-lipoic acid, deferoxamine mesylate, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), rotenone, acridine orange, ferrostatin-1, necrostatin-1, liproxstatin-1, Trolox, exo1, genistein, 2-deoxy-*D*-glucose (2DG), methyl-β-cyclodextrin (MβCD), chlorpromazine hydrochloride, amiloride hydrochloride, protease inhibitor cocktail (Cat# HY-K0010), and phosphatase inhibitor cocktail (Cat# HY-K0022) were purchased from MedChemExpress (Shanghai, China).

CAY10650 was purchased from Topscience (Shanghai, China).

An ATP assay kit (Cat# CK18), a cytotoxicity LDH assay kit (Cat# CK12), a cell counting kit-8 (CCK-8, Cat# CK04), FerroOrange (Cat# F374), JC-1 (Cat# MT09), MitoBright LT Deep Red (Cat# MT12), and Lipi-Blue (Cat# LD01) were purchased from Dojindo (Beijing, China).

A reduced GSH assay kit, a cysteine colorimetric assay kit, and a hematoxylin–eosin (H&E) stain kit were purchased from Nanjing Jiancheng Bioengineering Institute.

A cysteine fluorometric assay kit (Cat# ab211099), rabbit anti-ACSL4 (Cat# ab155282), and rabbit anti-GPX4 (Cat# ab125066) were purchased from Abcam (Shanghai, China).

Rabbit anti-FSP1 (Cat# 20886-1-AP) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Cat# SA00001-2) were purchased from Proteintech (Wuhan, China).

Rabbit anti-β-tubulin (Cat# ET1602-4) was purchased from HuaBio (Hangzhou, China).

Rabbit anti-SLC7A11 (Cat# 12691) and rabbit anti-γH2A.X (Cat# 9718) were purchased from Cell Signaling Technology, Inc.

Nystatin, Z-VAD-FMK, and dynasore were purchased from Selleck Chemicals LLC.

RIPA lysis buffer (Cat# K1120) was purchased from APExBIO (Houston, USA).

Tris-buffered saline powder (Cat# G0001-2L), SDS-PAGE running buffer powder (Cat# G2018-1L), PAGE transfer buffer powder (Cat# G2017-1L), and rabbit anti-Ki67 (Cat# GB111499) were purchased from Servicebio (Wuhan, China).

Trypsin (0.25 wt%) and ethylenediaminetetraacetic acid (EDTA, 0.02 wt%) in Puck's saline A (trypsin/EDTA solution) was purchased from Biological Industries (Sartorius Group, USA).

A malondialdehyde (MDA) assay kit was purchased from Abbkine (Wuhan, China).

The GSH and GSSG assay kit (Cat# RK05819) was purchased from ABclonal (Wuhan, China).

Fetal bovine serum (FBS), goat serum, recombinant human insulin, and phosphate-buffered saline (PBS) were purchased from VivaCell (Shanghai, China).

Normocin (Cat# ant-nr-2) was purchased from Invivogen (San Diego, USA).

SDS-PAGE sample loading buffer (Cat# P0015) and Tris-HCl buffer (pH=7.4, Cat# ST774) were purchased from Beyotime (Shanghai, China).

A PAGE gel fast preparation kit (Cat# PG112) was purchased from Epizyme (Shanghai, China).

Tris-buffered saline with Tween-20 (TBST), and Giemsa staining solution were purchased from Solarbio (Beijing, China).

Minimum essential medium (MEM, Cat# C11095500BT), Dulbecco's modified Eagle medium (DMEM, Cat# C11995500BT), Roswell Park Memorial Institute 1640 medium (RPMI-1640, Cat# C11875500CP), Hank's balanced salt solution (HBSS, Cat# C14175500BT), GlutaMAX (Cat# 35050061), CellEvent Caspase-3/7 Green (Cat# C10423), SYTO Deep Red (Cat# S34900), BODIPY 581/591 C11 lipid peroxidation sensor (C_{11}-BODIPY, Cat# D3861), MitoSOX Red mitochondrial superoxide indicator (Cat# M36008), Alexa Fluor 488 cadaverine (Cat# A30676), DyLight 594-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Cat# 35560), BCA protein assay kit (Cat# 23227), and PageRuler prestained protein ladder (10–180 kDa, Cat# 26616) were purchased from Thermo Fisher Scientific Inc.

Paraformaldehyde fixing solution was purchased from Babio (Jinan, China).

A chemiluminescence detection kit (Cat# E412-01) was purchased from Vazyme (Nanjing, China).

A polymer-enhanced two-step immunohistochemistry detection kit (Cat# PV-9001) and a diaminobenzidine kit (Cat# ZLI-9017) were purchased from ZSGB-BIO (Beijing, China).

Experimental instrumentation

The blue LED (peak wavelength, 448.4 nm; half bandwidth, 18.0 nm) used in the photochemical reactions and cell experiments was purchased from Ouying Lighting (Shenzhen, China). A blue laser light source (peak wavelength, 446.3 nm; half bandwidth, <2.5 nm) equipped with a 5 mm optical fiber collimator was purchased from Perfectlight (Beijing, China).

Liquid-state ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer.

^{13}C Cross-polarization magic-angle spinning solid-state NMR (^{13}C CP/MAS ssNMR) spectra were recorded on a Bruker Avance III HD 600 MHz NMR spectrometer with a 4 mm double-resonance MAS probe.

Supercritical carbon dioxide drying was performed using a Tousimis Samdri-PVT-3D critical point dryer. Damp solids were contained in folded filter paper secured with a staple and dried with a 15 min purge time and 15 min equilibration time after heating.

Powder X-ray diffraction (PXRD) patterns were obtained on a SmartLab SE X-ray powder diffractometer (Rigaku, Japan) with Cu *K*α line focused radiation (λ = 1.54056 Å) in the range of 2θ = 2.00°–50.00° at a step size of 0.01°.

Elemental analyses were performed on an Elementar Vario EL Cube CHNS elemental analyzer.

Samples were degassed under vacuum at 120°C for 8 h, and nitrogen-adsorption isotherms were measured at 77 K using a Micromeritics ASAP2020 HD88 surface area and porosity analyzer. The Brunauer–Emmett–Teller (BET) equation was used to calculate the specific surface areas.

High-resolution mass spectra (HRMS) were recorded on a Bruker SolariX 7 T Fourier transform ion cyclotron resonance mass spectrometer equipped with an electron spray ionization (ESI) source. All HRMS data were reported as mass per charge ratios (m/z).

Fourier transform infrared (FT-IR) spectra were obtained on a Thermo Scientific Nicolet iS50 FT-IR spectrometer equipped with a diamond attenuated total reflection (ATR) module between 4000–400 cm^{-1}. Each spectrum represented an average of 16 scans.

Inductively coupled plasma–mass spectrometry (ICP–MS) measurements were carried out using a PerkinElmer NexION 300X ICP–MS.

Transmission electron microscopy (TEM) images were recorded on a Hitachi HT7700 120 kV compact-digital instrument. The TEM samples were prepared in methanol by sonicating the material for 5 min, followed by application on a carbon-coated copper TEM grid (200 mesh), and air-dried at room temperature.

Scanning electron microscopy (SEM) images were recorded using a Hitachi SU8010 instrument. The SEM samples were prepared by depositing a diluted suspension onto silicon wafers (approximately 3 × 3 mm), followed by air drying and coating with a thin layer of Pt to increase the contrast.

X-ray photoelectron spectroscopy (XPS) profiles were obtained on a Thermo Fisher Scientific ESCALAB 250Xi XPS System equipped with a monochromatic Al *K*α radiation source (1486.6 eV).

The hydrodynamic particle size and zeta potential were measured using a Malvern Zetasizer Nano ZS90 system.

Laser scanning confocal fluorescence images of cells were captured with a Leica TCS SP8 confocal laser scanning microscope equipped with 405 nm, 458 nm, 488 nm, 514 nm, 561 nm, and 633 nm lasers. The imaging scan speed was 400 Hz, and transmitted light was used to find the areas of interest to reduce photodamage to the sample. Glass bottom dishes and 4/8-well chamber slides (Cellvis, USA) were used for cell culture in experiments. The original culture media and PBS were replaced with HBSS supplemented with HEPES (25 mM, pH 7.4) and GlutaMAX (1×) to provide better buffering capacity under normal CO_2 concentrations before live cell imaging.

Microplate assays were conducted using a Molecular Devices SpectraMax i3x multimode microplate detection system.

Western blot images were obtained using a GE Healthcare Amersham Imager 600 luminescent image analyzer.

Flow cytometric analysis was performed using a BD FACSCalibur flow cytometer.

Cell culture

The HCT-116 (human colorectal carcinoma) and HT-1080 (human fibrosarcoma) cell lines were provided by the Cell Bank, Chinese Academy of Sciences (Shanghai, China). The MCF-7 (human breast adenocarcinoma) cell line was provided by Institute of Basic Medicine, Shandong Academy of Medical Sciences (Jinan, China). The KYSE-150 (human esophageal squamous cell carcinoma), KYSE-510 (human esophageal squamous cell carcinoma), HEEC (human esophageal epithelia) cell lines were provided by Department of Radiation Oncology, Cheeloo College of Medicine, Qilu Hospital, Shandong University (Jinan, China). The MCF-10A (human mammary epithelia) cell line was provided by the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China).

HCT-116, KYSE-150, KYSE-510, and HEEC cells were cultured in RPMI-1640 medium supplemented with fetal bovine serum (10 vol%) and normocin (100 μg/mL). HT-1080 cells were cultured in MEM supplemented with fetal bovine serum (10 vol%) and normocin (100 μg/mL). MCF-7 cells were cultured in DMEM supplemented with fetal bovine serum (10 vol%), GlutaMAX (1×), human recombinantand insulin (10 μg/mL), and normocin (100 μg/mL). MCF-10A cells were cultured using MEGM BulletKit supplemented with normocin (100 μg/mL). All cells were cultured in a water-jacketed CO_2 incubator with CO_2 (5 vol%) at 37°C.

Synthesis of *S*-phenethyl-*L*-cysteine

The reaction mixture of *L*-cysteine hydrochloride monohydrate (0.70 g, 4.0 mmol), styrene (115 μL, 1.0 mmol), $Ru(bpz)_3(PF_6)_2$ (2.6 mg, 3.0 μmol), water (1.0 mL), and acetonitrile (1.0 mL) was added to a quartz tube sealed with a rubber stopper and stirred at 200 rpm for 3.5 h under blue LED illumination (20 mW/cm^2). The solvent was removed under reduced pressure. Then, the residue was recrystallized in water, washed with acetone, and dried at 60°C to obtain the desired compound.

Appearance: white solid. Yield: 205 mg (91%).

^{1}H NMR (400 MHz, DMSO-d_6/CD_3COOD, 5:1, v/v) δ/ppm 7.36–7.15 (m, 5H), 3.68 (dd, J = 7.6, 4.0 Hz, 1H), 3.06 (dd, J = 14.5, 4.0 Hz, 1H), 2.94 (dd, J = 14.5, 7.6 Hz, 1H), 2.88–2.73 (m, 4H).

^{13}C NMR (101 MHz, DMSO-d_6/CD_3COOD, 5:1, v/v) δ/ppm 170.21, 140.72, 128.90, 128.65, 126.53, 53.36, 35.63, 33.13, 32.62.

HRMS (ESI) m/z calculated for $C_{11}H_{16}NO_2S$ $[M+H]^+$: 226.08963; found: 226.08906. m/z calculated for $C_{11}H_{15}NO_2SNa$ $[M+Na]^+$: 248.07157; found: 248.07088.

ATR–FTIR ν/cm^{-1} 3084 (m), 3060 (m), 3003 (m), 2965 (m), 2935 (m), 2917 (m), 3028 (m), 1618 (m), 1578 (s), 1480 (s), 1453 (w), 1408 (s), 1341 (m), 1303 (m), 1278 (w), 1212 (w), 1128 (w), 1099 (w), 1074 (w), 1048 (w), 1028 (w), 1001 (w), 959 (w), 938 (w), 914 (w), 888 (w), 841 (m), 782 (w), 747 (w), 730 (m), 695 (s), 649 (w), 612 (w), 562 (w), 540 (m), 491 (w), 454 (m).

Synthesis of *S*-phenethyl-*L*-glutathione

The reaction mixture of GSH (1.23 g, 4.0 mmol), styrene (115 μL, 1.0 mmol), $Ru(bpz)_3(PF_6)_2$ (2.6 mg, 3.0 μmol), water (2.0 mL), and acetonitrile (2.0 mL) was added to a quartz tube sealed with a rubber stopper and stirred at 200 rpm for 3.5 h under blue LED illumination (20 mW/cm^2). The solvent was removed and the residue was recrystallized in water, washed with acetone, and dried at 60°C to obtain the desired compound.

Appearance: white solid. Yield: 224 mg (54%).

^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm 8.59 (d, J = 5.6 Hz, 1H), 8.43 (d, J = 8.6 Hz, 1H), 7.36–7.12 (m, 5H), 4.49 (td, J = 8.9, 4.6 Hz, 1H), 3.72 (s, 2H), 3.42 (t, J = 6.6 Hz, 1H), 2.93 (dd, J = 13.7, 4.6 Hz, 1H), 2.86–2.73 (m, 4H), 2.68 (dd, J = 13.7, 9.5 Hz, 1H), 2.33 (t, J = 7.8 Hz, 2H), 1.93 (q, J = 7.4 Hz, 2H).

^{13}C NMR (101 MHz, DMSO-d_6) δ/ppm 172.25, 171.51, 171.21, 171.18, 140.95, 128.97, 128.73, 126.56, 53.57, 52.93, 41.75, 35.84, 34.00, 33.17, 31.95, 27.33.

HRMS (ESI) m/z calculated for $C_{18}H_{26}N_3O_6S$ $[M+H]^+$: 412.15368; found: 412.15304. m/z calculated for $C_{18}H_{25}N_3O_6SNa$ $[M+Na]^+$: 434.13563; found: 434.13509.

ATR–FTIR ν/cm^{-1} 3372 (m), 3344 (m), 3028 (m), 2952 (m), 1671 (s), 1644 (s), 1511 (s), 1455 (w), 1431 (m), 1351 (s), 1309 (s), 1251 (m), 1227 (s), 1133 (m), 1080 (m), 1030 (w), 964 (w), 909 (w), 885 (w), 819 (w), 800 (w), 717 (m), 695 (s), 644 (m), 611 (m), 564 (m), 545 (s), 510 (w), 458 (m), 414 (m).

Synthesis of COF-V-Cys

A mixture of *L*-cysteine hydrochloride monohydrate (351 mg, 2.0 mmol), Ru(II)@COF-V (180 mg), and PBS (50 mL) was added to a quartz tube sealed with a rubber stopper and stirred at 200 rpm for 0.5 h under blue LED irradiation (20 mW/cm^2). The precipitate was collected by centrifugation at 12000 rpm for 0.5 h at 4°C, washed three times with water, and then washed three times with ethanol. Finally, the powders were dried in supercritical carbon dioxide.

Synthesis of COF-V-GSH

A mixture of GSH (614 mg, 2.0 mmol), Ru(II)@COF-V (180 mg), and PBS (50 mL) was added to a quartz tube sealed with a rubber stopper and stirred at 200 rpm for 0.5 h under blue LED irradiation (20 mW/cm^2). The precipitate was collected by centrifugation at 12000 rpm for 0.5 h at 4°C, washed three times with water, and then washed three times with ethanol. Finally, the powders were dried in supercritical carbon dioxide.

Reaction kinetics

Ru(II)@COF-V (20 mg) was dispersed in PBS (50 mL) containing cysteine (0.1 mM) or GSH (10 mM) and exposed to a blue LED light (20 mW/cm^2) for 2 h at 37°C. During the illumination period, 1 mL of the suspension was taken and centrifuged at 13300 rpm for 10 min at 25°C. The obtained supernatants were used for cysteine and GSH detection using the corresponding quantitative assay kits.

Cellular uptake and colocalization experiments

HCT-116 cells cultured in 8-well chamber slides were pretreated with oleic acid (200 μL, 200 μM) for 12 h, triacsin C (200 μL, 5.0 μM) for 12 h, and CAY10650 (200 μL, 20 nM) for 1 h. The pretreated and untreated HCT-116 cells were cultured with COF-V-AF488 (400 μL, 100 μg/mL) for 2 h in a CO_2 incubator and washed

twice with PBS. The cells were loaded with Lipi-Blue (200 μL, 0.1 μM) for 15 min in a CO_2 incubator. Laser scanning confocal fluorescence images were captured. The green images of COF-V-AF488 were recorded at an excitation wavelength of 488 nm and an emission wavelength range of 500–540 nm. The red images of Lipi-Blue were recorded at an excitation wavelength of 405 nm and an emission wavelength range of 430–470 nm.

HT-1080, MCF-7, KYSE-510, KYSE-150, HEEC, and MCF-10A cells cultured in 4-well glass-bottom dishes were cultured with COF-V-AF488 (400 μL, 100 μg/mL) for 2 h in a CO_2 incubator and washed twice with PBS. The cells were loaded with Lipi-Blue (200 μL, 0.1 μM) for 15 min (HT-1080, MCF-7, KYSE-510, and KYSE-150 cells) or 30 min (HEEC and MCF-10A cells) in a CO_2 incubator. Laser scanning confocal fluorescence images were captured. The green images of COF-V-AF488 were recorded at an excitation wavelength of 488 nm and an emission wavelength range of 500–540 nm. The red images of Lipi-Blue were recorded at an excitation wavelength of 405 nm and an emission wavelength range of 430–470 nm.

Cellular uptake mechanism

To investigate the relationship between lipid droplet content and cellular uptake, the following experiments were performed. HCT-116 cells cultured in 6-well plates were pretreated with oleic acid (1.0 mL, 200 μM) for 12 h, triacsin C (1.0 mL, 2.0 μM) for 12 h, and CAY10650 (1.0 mL, 20 nM) for 1 h. The pretreated and untreated HCT-116 cells were cultured with COF-V-AF488 (1.0 mL, 100 μg/mL) for 2 h in a CO_2 incubator and washed twice with PBS. The cells were collected using a trypsin/EDTA solution, resuspended in HBSS, strained through a 70 μm cell strainer, and analyzed using a flow cytometer equipped with a 488 nm laser for excitation and an FL1 channel for collection. Untreated cells were used as the control group.

To investigate the role of energy-dependent pinocytosis in cellular uptake, the following experiments were performed. HCT-116 cells cultured in 6-well plates were pretreated with 2DG (1.0 mL, 150 mM), dynasore (1.0 mL, 75 μM), nystatin (1.0 mL, 25 μM), MβCD (1.0 mL, 10 mg/mL), genistein (1.0 mL, 40 μM), chlorpromazine (1.0 mL, 50 μM), or amiloride (1.0 mL, 0.2 mM) for 1 h. The pretreated and untreated HCT-116 cells were cultured with COF-V-AF488 (1.0 mL, 200 μg/mL) for 2 h in a CO_2 incubator and washed twice with PBS. The cells were harvested using a trypsin/EDTA solution, resuspended in HBSS, strained through a 70 μm cell strainer, and analyzed using a flow cytometer equipped with a 488 nm laser for excitation and an FL1 channel for collection. Untreated cells were used as the control group.

Propidium iodide staining assay

HCT-116 cells cultured in 8-well chamber slides were treated with Ru(II)@COF-V (400 μL, 0 or 400 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After an additional 24 h of incubation, the cells were incubated with a mixture (200 μL) of SYTO Deep Red (2.0 μM) and propidium iodide (3.0 μM) for 30 min in a CO_2 incubator and washed twice with PBS. Then, laser scanning confocal fluorescence images were captured. The green images of SYTO Deep Red were detected by excitation at 633 nm and emission at 650–690 nm. The red images of propidium iodide were detected by excitation at 488 nm and emission at 590–620 nm. Untreated cells were used as the negative control group, and cells treated with ethanol for 30 min at 25°C were used as the positive control group.

CCK-8 cell viability assays

HCT-116, HT-1080, MCF-7, KYSE-510, KYSE-150, HEEC, and MCF-10A cells cultured in 96-well plates

were treated with Ru(II)@COF-V (100 μL, 0–400 μg/mL) for 2 h in a CO_2 incubator. The cells were exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min and incubated in a CO_2 incubator for 24 h. Subsequently, the cells were washed three times with PBS. Then, PBS (90 μL) and CCK-8 solution (10 μL) were added to each well, and the plate was incubated in a CO_2 incubator for approximately 1 h. The absorbance at 450 nm was measured using a multimode microplate detection system. Untreated cells were used as the control group, while cells treated with methanol for 1 h at 25°C were used as the blank.

Clonogenic assay

HCT-116 cells cultured in 6-well plates were treated with Ru(II)@COF-V (2.0 mL, 0–400 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After approximately 7 days of incubation, the cells were fixed with paraformaldehyde (2.0 mL, 4 wt%) for 2 h and stained with fresh Giemsa staining solution for 1 h at room temperature. The plates were washed with water, air-dried, and photographed with a digital camera. The untreated well was used as the control group.

Intracellular cysteine, GSH, GSSG, and MDA measurements

HCT-116 cells were cultured in 6-, 24-, and 96-well plates, treated with Ru(II)@COF-V (1.0, 0.5, or 0.1 mL; 0 or 200 μg/mL) for 2 h in a CO_2 incubator, and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After an additional 30 min or 24 h of incubation, the cells were carefully rinsed with PBS and used for cysteine, GSH, GSSG, and MDA measurements according to the manufacturer's guidelines. The cysteine, GSH, GSSG, and MDA contents were normalized to the total protein amount quantified using a BCA protein assay kit. Untreated cells were used as the control group.

Intracellular ROS detection

HCT-116 cells cultured in 8-well chamber slides were treated with Ru(II)@COF-V (400 μL, 0 or 100 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After an additional 24 h of incubation, the cells were loaded with DCFH-DA (200 μL, 20 μM) for 30 min in a CO_2 incubator and then washed twice with PBS. Laser scanning confocal fluorescence images were captured with the green signal recorded at an excitation wavelength of 488 nm and an emission wavelength range of 500–540 nm. Untreated cells were used as the control group.

For ROS staining rescue experiments, during the 24 h incubation after blue LED irradiation, the medium was supplemented with one of the following compounds: ferrostatin-1 (1.0 μM), Z-VAD-FMK (50 μM), *N*-acetylcysteine (1.0 mM), GSH-OEt (1.0 mM), α-lipoic acid (50 μM), or deferoxamine mesylate (100 μM).

Lipid peroxidation detection

HCT-116 cells cultured in 8-well chamber slides were treated with Ru(II)@COF-V (400 μL, 0 or 100 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After an additional 24 h of incubation, the cells were loaded with C_{11}-BODIPY (200 μL, 2.0 μM) for 30 min in a CO_2 incubator and then washed twice with PBS. Laser scanning confocal fluorescence images were captured. The green signal of the oxidized C_{11}-BODIPY was recorded at an excitation wavelength of 488 nm and an emission wavelength range of 490–530 nm. The red signal of the reduced C_{11}-BODIPY was recorded at an excitation wavelength of 561 nm and an emission wavelength range of 570–610 nm. Untreated cells were used as the control group.

For lipid peroxide staining rescue experiments, during the 24 h incubation after blue LED irradiation, the medium was supplemented with one of the following compounds: ferrostatin-1 (1.0 μM), Z-VAD-FMK (50 μM), *N*-acetylcysteine (1.0 mM), GSH-OEt (1.0 mM), α-lipoic acid (50 μM), and deferoxamine mesylate (100 μM).

Mitochondrial membrane potential measurement

HCT-116 cells cultured in 8-well chamber slides were treated with Ru(II)@COF-V (400 μL, 0 or 100 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After an additional 0.5–48 h of incubation, the cells were incubated with JC-1 (200 μL, 2.0 μM) for 20 min in a CO_2 incubator and washed twice with PBS. Laser scanning confocal fluorescence images were captured. The green signal of the JC-1 monomer was detected by excitation at 488 nm and emission at 500–550 nm. The red signal of *J*-aggregated JC-1 was detected by excitation at 561 nm and emission at 570–620 nm. Untreated cells were used as the control group.

Intracellular ATP detection

HCT-116 cells cultured in white 96-well plates were treated with Ru(II)@COF-V (200 μL, 200 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED (0 or 20 mW/cm^2) for 15 min. After an additional 12 h of incubation, the cells were carefully rinsed with PBS and used to measure ATP according to the manufacturer's guidelines.

Mitochondrial superoxide detection

HCT-116 cells were treated with Ru(II)@COF-V (1.0 mL, 0 or 100 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After an additional 0.5–24 h of incubation, the cells were harvested by a trypsin/EDTA solution, resuspended in PBS containing MitoSOX Red (1.0 mL, 10 μM), and incubated for 30 min in a CO_2 incubator. Subsequently, the cells were resuspended in HBSS, strained through a 70 μm cell strainer, and analyzed using a flow cytometer equipped with a 488 nm laser for excitation and the FL2 channel for collection. Untreated cells were used as the control group.

Mitochondrial morphology

HCT-116 cells cultured in 4-well glass-bottom dishes were incubated with MitoBright LT Deep Red (200 μL, 100 nM) for 30 min in a CO_2 incubator. Subsequently, the cells were treated with Ru(II)@COF-V (500 μL, 0 or 100 μg/mL) for 2 h in a CO_2 incubator, exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min, and washed twice with PBS carefully. After an additional 0.5–48 h of incubation, laser scanning confocal fluorescence images were captured. The red signal was detected by excitation at 633 nm and emission at 650–700 nm. Untreated cells were used as the control group.

Intracellular Fe^{2+} detection

HCT-116 cells cultured in 8-well chamber slides were treated with Ru(II)@COF-V (400 μL, 0 or 100 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. The cells were treated with deferoxamine mesylate (400 μL, 0 or 100 μM) for 24 h and then loaded with FerroOrange (200 μL, 1.0 μM) for 30 min in a CO_2 incubator. Laser scanning confocal fluorescence images were captured with the orange signal recorded at an excitation wavelength of 561 nm and an emission wavelength range of 570–620 nm. Untreated cells were used as the control group.

Lysosomal membrane permeability assay

HCT-116 cells cultured in 8-well chamber slides were treated with Ru(II)@COF-V (400 μL, 0 or 100 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After an additional 24 h of incubation, the cells were loaded with acridine orange (200 μL, 20 μM) for 10 min in a CO_2 incubator and then washed twice with PBS. Laser scanning confocal fluorescence images were captured. The green images of deprotonated acridine orange were excited by 488 nm light, and the emission wavelength range was collected at 510–550 nm. The red images of protonated acridine orange were excited by 488 nm light, and the emission wavelength range was collected at 620–660 nm. Untreated cells were used as the control group.

Western blotting

HCT-116 cells cultured in 6-well plates were treated with Ru(II)@COF-V (1.0 mL, 0 or 200 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After 24 h of additional culture, the adherent cells were washed once with ice-cold PBS and lysed in ice-cold RIPA lysis buffer (APExBIO, Cat# K1120) supplemented with protease inhibitor cocktail (MedChemExpress, Cat# HY-K0010) and phosphatase inhibitor cocktail (MedChemExpress, Cat# HY-K0022). The lysates were centrifuged at 13000 rpm at 4°C for 15 min, and the supernatant was isolated to exclude debris. The protein concentration was quantified using a BCA protein assay kit (Thermo Fisher, Cat# 23227). The samples were prepared with SDS–PAGE sample loading buffer (Beyotime, Cat# P0015) at 95°C for 15 min, and equal amounts of protein per sample were loaded onto PAGE gels (Epizyme, Cat# PG112) for electrophoresis to separate the target protein. After transferring the protein to a polyvinylidene difluoride membrane and blocking for 1 h using nonfat powdered milk (5 wt%) in TBST, the membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies used here were rabbit anti-FSP1 (1:1000 dilution, Proteintech, Cat# 20886-1-AP), rabbit anti-SLC7A11 (1:1000 dilution, Cell Signaling Technology, Cat# 12691), rabbit anti-ACSL4 (1:10000 dilution, Abcam, Cat# ab155282), rabbit anti-GPX4 (1:1000 dilution, Abcam, Cat# ab125066), and rabbit anti-β-tubulin (1:1000 dilution, HuaBio, Cat# ET1602-4). Subsequently, the membranes were washed three times for 10 min in TBST and incubated with HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:10000 dilution, Proteintech, Cat# SA00001-2) for 1 h at 25°C. The protein of interest was observed using a chemiluminescence detection kit (Vazyme, Cat# E412-01).

Cell death rescue experiments

HCT-116 cells cultured in 96-well plates were treated with Ru(II)@COF-V (100 μL, 0–200 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (20 mW/cm^2) for 15 min. The cells were incubated in a CO_2 incubator for 24 h in the presence of Trolox (100 μL, 1.0 mM), *N*-acetylcysteine (100 μL, 1.0 mM), 2-mercaptoethanol (100 μL, 1.0 mM), GSH-OEt (100 μL, 1.0 mM), α-lipoic acid (100 μL, 50 μM), deferoxamine mesylate (100 μL, 100 μM), liproxstatin-1 (100 μL, 50 nM), ferrostatin-1 (100 μL, 1.0 μM), Z-VAD-FMK (100 μL, 50 μM), necrostatin-1 (100 μL, 0.5 μM), 3-methyladenine (100 μL, 50 μM), belnacasan (100 μL, 20 μM), rotenone (100 μL, 0–200 nM), or necrox-5 (100 μL, 0–8.0 μM). Subsequently, the cells were washed three times with PBS. Then, PBS (90 μL) and CCK-8 solution (10 μL) were added to each well, and the plate was incubated in a CO_2 incubator for approximately 1 h. The absorbance at 450 nm was measured using a multimode microplate detection system. Untreated cells were used as the control group, while cells treated with methanol were used as the blank.

γH2A.X immunofluorescence staining assay

HCT-116 cells cultured in glass-bottom dishes were treated with Ru(II)@COF-V (400 μL, 0 or 200 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After an additional 2 h of incubation, the cells were fixed in paraformaldehyde (400 μL, 4 wt%) for 20 min and washed three times with PBS. The cells were permeabilized with Triton X-100 (400 μL, 0.5 vol%) for 5 min and washed twice with PBS. The cells were incubated in PBS (400 μL) containing normal goat serum (10 vol%) and Tween-20 (0.1 vol%) for 1 h at room temperature. The cells were incubated with rabbit anti-γH2A.X (Ser139) primary antibody (100 μL, 1:400 dilution, Cell Signaling Technology, Cat# 9718) at 4°C overnight and washed three times with PBS. The cells were incubated with DyLight 594-conjugated goat anti-rabbit IgG (H+L) secondary antibody (200 μL, 1:500 dilution, Thermo Fisher, Cat# 35560) for 1 h at room temperature. Finally, the cell nuclei were counterstained with Hoechst 33342 (400 μL, 20 μM) for 30 min and washed twice with PBS. Laser scanning confocal fluorescence images were captured. The red images of DyLight 594 were recorded at an excitation wavelength of 561 nm and an emission wavelength range of 600–650 nm. The blue signals of Hoechst 33342 were recorded at an excitation wavelength of 405 nm and an emission wavelength range of 420–450 nm. Untreated cells were used as the negative control group. Cells treated with staurosporine (400 μL, 2.0 μM) for 12 h and cells treated with cisplatin (400 μL, 30 μM) for 12 h were used as the positive control groups.

Caspase 3 activation assay

HCT-116 cells cultured in 8-well chamber slides were treated with Ru(II)@COF-V (400 μL, 0 or 200 μg/mL) for 2 h in a CO_2 incubator and exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min. After an additional 24 h of incubation, the cells were loaded with Hoechst 33342 (400 μL, 20 μM) for 30 min and then incubated with CellEvent Caspase-3/7 Green for 30 min in a CO_2 incubator. Laser scanning confocal fluorescence images were captured. The green signals were recorded at an excitation wavelength of 488 nm and an emission wavelength range of 500–540 nm. The red images of Hoechst 33342 were excited by 405 nm light, and the emission wavelength range was collected at 420–450 nm. Untreated cells were used as the negative control group. Cells treated with staurosporine (400 μL, 2.0 μM) for 24 h and cells treated with cisplatin (400 μL, 30 μM) for 24 h were used as the positive control groups.

Antitumor therapy in multicellular tumor spheroids

Several 24-well plates were coated with an ethanol solution of poly(2-hydroxyethyl methacrylate) (1.0 mL, 12 mg/mL) and exposed to ultraviolet light for 24 h. HCT-116 cells were seeded into the coated 24-well plates and cultured in a CO_2 incubator until multicellular tumor spheroids formed. Multicellular tumor spheroids with less than 10% size variation between spheroids were selected under a microscope and transferred to another coated 24-well plate for subsequent experiments.

To evaluate the penetration of COF-V in multicellular tumor spheroids, HCT-116 tumor spheroids with a size of approximately 400 μm were pretreated with exo1 (500 μL, 50 μM), dynasore (500 μL, 75 μM), genistein (500 μL, 40 μM), and chlorpromazine (500 μL, 50 μM) for 2 h in a CO_2 incubator. The treated and untreated multicellular tumor spheroids were cultured with COF-V-AF488 (500 μL, 200 μg/mL) for 4 h in a CO_2 incubator and transferred to an 8-well chamber slide. Laser scanning confocal tomography fluorescence images were captured at intervals of 20 μm from the bottom to top of the HCT-116 multicellular tumor spheroids upward. The green images of COF-V-AF488 were recorded at an excitation wavelength of 488 nm

and an emission wavelength range of 500–540 nm.

To evaluate antitumor efficiency in multicellular tumor spheroids, HCT-116 tumor spheroids with a size of approximately 200 μm were treated with Ru(II)@COF-V (500 μL, 0 or 200 μg/mL) for 4 h in a CO_2 incubator. The tumor spheroids were exposed to a blue LED light (0 or 20 mW/cm^2) for 15 min and incubated in a CO_2 incubator for 3–7 days. Subsequently, the tumor spheroids were photographed and then collected by centrifugation at 1000 rpm for 10 min. The collected tumor spheroids were lysed in lysis buffer followed by MDA and total protein measurements according to the manufacturer's guidelines. The obtained supernatants were used for LDH measurement according to the manufacturer's guidelines. Untreated tumor spheroids were used as the control group. The MDA contents were normalized to the total protein amount. The LDH release amounts were expressed as a percentage relative to the lysed tumor spheroids treated with Triton X-100 (1.0 vol%).

For rescue experiments of tumor spheroids, HCT-116 tumor spheroids with a size of approximately 200 μm were treated with Ru(II)@COF-V (1.0 mL, 200 μg/mL) for 4 h in a CO_2 incubator. The tumor spheroids were exposed to a blue LED light (20 mW/cm^2) for 15 min and incubated in a CO_2 incubator for 3–7 days in the presence of *N*-acetylcysteine (1.0 mL, 1.0 mM), ferrostatin-1 (1.0 mL, 1.0 μM), necrostatin-1 (1.0 mL, 0.5 μM), Z-VAD-FMK (1.0 mL, 50 μM), and 3-methyladenine (1.0 mL, 50 μM). The tumor spheroids were photographed. Untreated tumor spheroids were used as the control group.

***In vivo* antitumor experiments**

BALB/c nude mice (aged 4 weeks, female) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The nude mice were housed in a filter-topped facility (pathogen-free) on autoclaved food and water and kept in a room on a 12:12 h light-dark cycle with a temperature between 20–23°C and 30–70% relative humidity.

HCT-116 cells (approximately 5×10^6 cells) suspended in HBSS (50 μL) were subcutaneously injected into the flanks of each nude mouse to establish the HCT-116 xenograft model. The length (L) and width (W) of the tumor were determined using digital calipers. The tumor volume (V) was calculated using the formula $V = LW^2/2$. Nude mice that failed to develop tumors from the beginning of the experiment were excluded.

When the tumor size reached 100–150 mm^3, the mice were randomly divided into 5 groups. Group 1, as a control group, was intratumorally injected with PBS (50 μL) at days 0, 3, 6, and 9. Group 2 was intratumorally injected with PBS (50 μL) at days 0, 3, 6, and 9, followed by exposure to three 5:5 min on-off cycles of blue laser illumination (0.2 W/cm^2). Group 3 was intratumorally injected with Ru(II)@COF-V (50 μL, 1.0 mg/mL) at days 0, 3, 6, and 9. Group 4 was intratumorally injected with Ru(II)@COF-V (50 μL, 1.0 mg/mL) at days 0, 3, 6, and 9, followed by exposure to three 5:5 min on-off cycles of blue laser illumination (0.2 W/cm^2). Group 5 was peritumorally injected with liproxstatin-1 (5.0 mg/kg) at days 0, 3, 6, and 9, in addition to the same treatment as group 4.

At day 4, four mice in each group were randomly selected and euthanized. The harvested tumor tissues were washed with PBS, cut into small pieces, and homogenized in extraction buffer at 4°C. The tumor homogenates were centrifuged at 10000 rpm for 15 min at 4°C, and the supernatants were used for measurements of MDA and total protein contents according to the manufacturers' guidelines of the assay kits. The data were normalized to the total protein content.

The remaining mice continued to be reared. When the tumor size reached 15 mm in either dimension, the *in vivo* antitumor experiment was terminated, and all mice were euthanized. The tumor tissues and major organs (*i.e.*, heart, liver, spleen, lung, and kidney) were collected and fixed in paraformaldehyde (4 wt%) for histological analysis.

The fixed tumor tissues and major organs were dehydrated and embedded in paraffin following routine methods. The paraffin-embedded tissues were cut in to slices with a thickness of 3–4 μm, deparaffinized, and rehydrated. H&E staining of the slices was performed using an H&E stain kit according to the manufacturer's guidelines. Furthermore, immunohistochemical staining of the slices was performed using a polymer-enhanced two-step immunohistochemistry detection system (ZSGB-BIO, Cat# PV-9001) and a diaminobenzidine kit (ZSGB-BIO, Cat# ZLI-9017) following the manufacturer's protocols. The antibodies used in immunohistochemical analysis are rabbit anti-GPX4 (1:100 dilution, Abcam, Cat# ab125066) and rabbit anti-Ki67 (1:1200 dilution, Servicebio, Cat# GB111499).