Supporting Information

In situ synthesis of bioorthogonally catalyzed drugs based on hydrogen-bonded organic frameworks for cancer therapy†

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Experimental Section

Chemicals and Materials. *N, N*-Dimethylformamide (C_3H_7NO, AR, 99.5%, Shanghai Macklin Biochemical Co. Ltd), *N*-Methyl Pyrrolidone (C_5H_9NO, 99%, Shanghai Macklin Biochemical Co. Ltd), Copper(II) tetra-(4-carboxyphenyl) porphyrin ($C_{48}H_{24}CuN_4O_8$, 95%, Jilin Chinese Academy of Sciences-Yanshen Technology Co. Ltd), 5-ethynyl-2-methoxyaniline (C_9H_9NO, 99.67%, Jilin Chinese Academy of Sciences-Yanshen Technology Co. Ltd), 5-azido-1,2,3-trimethoxybenzene ($C_9H_{11}N_3O_3$, 98.83%, Jilin Chinese Academy of Sciences-Yanshen Technology Co. Ltd), 3-Azido-7-hydroxycoumarin ($C_9H_5N_3O_3$, 98%, Shanghai Aladdin Biochemical Technology Co. Ltd), Phenylacetylene (C_8H_6, 97%, Shanghai Aladdin Biochemical Technology Co. Ltd), Hyaluronic acid (97%, Shanghai Macklin Biochemical Co. Ltd), Ethanol (99.8%, Shanghai Macklin Biochemical Co. Ltd), Tween 85 (Shanghai Macklin Biochemical Co. Ltd), Citric Acid ($C_6H_8O_7$, 99.5%, Shanghai Macklin Biochemical Co. Ltd). Dimethyl sulfoxide (C_2H_6SO, 99.9 %, Shanghai Macklin Biochemical Co. Ltd), Acetonitrile (C_2H_3N, 99.9 %, Shanghai Macklin Biochemical Co. Ltd), Glutathione reduced ($C_{10}H_{17}N_3O_6S$, 99 %, Shanghai Macklin Biochemical Co. Ltd).

Characterization. Powder X-ray diffraction (PXRD) patterns were obtained on a Rigaku MiniFlex 600 diffractometer with graphite monochromatized CuKα radiation (λ = 0.15405 nm). The sample was scanned at a scanning rate of 5/min in the 2θ range from 3 to 20° at room temperature. A field emission scanning electron microscope (Thermo Fisher, Apero 2S) was used to characterize the morphology of the sample. The UV-vis adsorption spectral values were collected on a UV-2600 spectrophotometer (Shimadzu). Dynamic light scattering (DLS) experiment was performed on Malvern Zeta Sizer-Nano ZS instrument at 25°C. MTT experiments were carried out using a microplate reader (Cytation5). The flow cytometry data was obtained by BD FACSymphony A1 Cell Analyzer, HPLC test and analysis were conducted on the LC-20 instrument, ^{1}H NMR spectrum was recorded on a Bruker-400 MHz NMR instrument, X-ray photoelectron Spectroscopy (XPS) spectra were analyzed by Thermo Fisher Scientific NEXSA Spectrometer Electron Spectroscopy (America).

Synthesis of Cu-HOF. Cu-TCPP (1mg, 0.0011 mmol) was dissolved in 0.3 mL of *N, N*-dimethylformamide (DMF) and 0.2 mL of *N*-methyl-2-pyrrolidone (NMP), and 0.042 mL of Tween 85 was then added. 2 mL of H_2O was introduced and stirred at room temperature for 5 min. 1.6 mL

of ethanol and 0.03 mL of citric acid (in 5 mg mL^{-1} of EtOH) were finally added and stirred for another 25 min. The precipitate was collected by centrifugation and washed three times with ethanol.

Synthesis of 1+2@Cu-HOF. Cu-TCPP (1 mg, 0.0011 mmol), 5-ethynyl-2-methoxyaniline (1mg, 0.0068 mmol) and 5-azido-1,2,3-trimethoxybenzene (1mg, 0.0048 mmol) were dissolved in 0.3 mL of *N, N*-dimethylformamide (DMF) and 0.2 mL of *N*-methyl-2-pyrrolidone (NMP), and 0.042 mL of Tween 85 was then added. 2 mL of H_2O was introduced and stirred at room temperature for 5 min. 1.6 mL of ethanol and 0.03 mL of citric acid (in 5 mg mL^{-1} of EtOH) were finally added and stirred for another 25 min. The precipitate was collected by centrifugation and washed three times with ethanol.

Synthesis of Cu-HOF@HA. 2 mg of **Cu-HOF** was dispersed in 4 mL of deionized water and then 2 mg of HA was added. After stirring for 24 h, the product was collected by centrifugation and washed with deionized water three times.

Synthesis of 1+2@Cu-HOF@HA. 2 mg of **1+2@Cu-HOF** was dispersed in 4 mL of deionized water and then 2 mg of HA was added. After stirring for 24 h, the product was collected by centrifugation and washed with deionized water three times.

Synthesis of triazole 3. The synthesis was carried out with the following conditions: the overall volume was 5mL (H_2O: t-ButOH=1:1), containing a solution of alkyne, 1, (1eq) and azide, 2, (1 eq), followed by the addition of copper (II) sulfate pentahydrate (0.1eq) and sodium ascorbate (0.2 eq) The resulting reaction was vigorously stirred for 4 h at 25℃. After obtaining the mixture, it was extracted with ethyl acetate. After obtaining the target product 3, it was vacuum-dried. **^{1}H NMR** (500 MHz, DMSO-d_6) δ 9.12 (s, 1H), 7.29 (d, J = 2.1 Hz, 1H), 7.27 (s, 2H), 7.09 (dd, J = 8.2, 2.1 Hz, 1H), 6.89 (d, J = 8.3 Hz, 1H), 4.88 (s, 2H), 3.90 (s, 7H), 3.81 (s, 3H), 3.72 (s, 4H). **^{13}C NMR** (126 MHz, DMSO) δ 153.55, 147.96, 146.56, 138.00, 137.20, 132.68, 123.05, 118.57, 113.76, 110.74, 110.72, 97.77, 60.23, 56.32, 55.38. **HRMS** (ESI) m/z calcd for $([C_{18}H_{20}N_4O_4]^+ H)^+$ 357.1567, found 257.1551

***In vitro* cytotoxicity of Cu-HOF@HA.** To study the *In vitro* biocompatibility of **Cu-HOF@HA**, L929 and HeLa cells were seeded separately into 96-well plates at a density of 6000 cells per well in 180 μL of culture medium. The plates were incubated for 24 h in a humidified incubator at 37 °C with 5% CO_2. After incubation, the culture medium was removed and replaced with **Cu-HOF@HA** solutions at various concentrations. For L929 cells, the initial concentration was 100 μg mL^{-1}, followed by serial two-fold dilutions. For HeLa cells, the maximum concentration used was 75 μg mL^{-1}, followed by serial two-fold dilutions starting from 50 μg mL^{-1}. The control group received fresh medium without **Cu-HOF@HA**. Each concentration was tested in six parallel wells. Subsequently, 20 μL of MTT reagent (5 mg mL^{-1}) was added to each well under dark conditions along with fresh culture medium, and the plates were incubated in the dark to allow the reaction to proceed. The MTT reagent is metabolized by mitochondrial succinate dehydrogenase in viable cells to form water-insoluble purple formazan crystals, which can be solubilized in DMSO. After 4 h of incubation, the supernatant was carefully removed, leaving the formazan crystals at the bottom of each well. Then, 150 μL of DMSO was added to each well, and the plates were gently shaken to

fully dissolve the crystals and ensure uniform distribution. The absorbance was measured at 630 nm using a multifunctional microplate reader.

***In vitro* cytotoxicity of Prodrug 1, Prodrug 2, and 1+2.** The *In vitro* cytotoxicity of Prodrug 1, Prodrug 2, and the combination of Prodrug 1+2 was assessed using the MTT assay in HeLa and 3T3 cells. HeLa and 3T3 cells were seeded separately into 96-well plates at a density of 6000 cells per well in 180 μL of culture medium. The plates were incubated for 24 h in a humidified incubator at 37 °C with 5% CO_2. After incubation, the original culture medium was removed, and replaced with fresh media containing varying concentrations of Prodrug 1, Prodrug 2, or their combination (1+2). All compounds were diluted using a two-fold serial dilution method starting from 100 μM. Wells in the control group were supplied with fresh medium without prodrugs. Each concentration was tested in six replicate wells. After 24 h of incubation, the cells were washed twice with PBS to remove residual compounds. Subsequently, under dark conditions, 20 μL of MTT solution (5 mg mL^{-1}) was added to each well along with fresh culture medium, and the plates were incubated in the dark to allow the reaction to occur. MTT is metabolized by mitochondrial succinate dehydrogenase in viable cells to generate insoluble purple formazan crystals, which were subsequently solubilized in DMSO. Following 4 h of incubation, the supernatant was carefully removed while retaining the formazan crystals at the bottom of each well. Then, 150 μL of DMSO was added to each well, and the plates were gently shaken to fully dissolve the crystals. Absorbance was measured at 630 nm using a multifunctional microplate reader.

Cellular Internalization of RhB@Cu-HOF@HA. Cu-TCPP (1 mg, 0.0011 mmol) and rhodamine B (RhB)-loaded (1 mg, 0.00209 mmol) were dissolved in 0.3 mL of *N, N*-dimethylformamide (DMF) and 0.2 mL of *N*-methyl-2-pyrrolidone (NMP), and 0.042 mL of Tween 85 was then added. 2 mL of H_2O was introduced and stirred at room temperature for 5 min. 1.6 mL of ethanol and 0.03 mL of citric acid (in 5 mg mL^{-1} of EtOH) were finally added and stirred for another 25 min. The precipitate was collected by centrifugation and washed three times with ethanol. 1 mg of HA was then added. After stirring for 24 h, the product was collected by centrifugation and washed with deionized water three times. HeLa cells were incubated with rhodamine B (RhB)-loaded **Cu-HOF@HA** (20 μg mL^{-1}) for 1 h, 4 h, 12 h, respectively. It was then washed with PBS for three times. The nuclei were labeled with 4,6-diamino-2-phenylindole (DAPI) for 10 min and then observed under confocal laser scanning microscopy (CLSM) images.

***In vitro* cytotoxicity evaluation of 1+2@Cu-HOF@HA.** The *In vitro* cytotoxicity of **1+2@Cu-HOF@HA** was evaluated using the MTT assay in HeLa cells. HeLa cells were seeded into 96-well plates at a density of 6000 cells per well in 180 μL of culture medium. The plates were incubated for 24 h in a humidified incubator at 37 °C with 5% CO_2. After incubation, the culture medium was removed and replaced with fresh medium containing various concentrations of **1+2@Cu-HOF@HA**. The test solutions were prepared using a two-fold serial dilution starting from 40 μg mL^{-1}. The control group received fresh medium without the compound. Each concentration was tested in six replicate wells. Following a 24 h incubation period, cells were washed twice with PBS to remove residual particles. Then, under dark conditions, 20 μL of MTT solution (5 mg mL^{-1}) was added to each well along with fresh culture medium. The plates were incubated in the dark to allow the MTT to be metabolized by mitochondrial succinate dehydrogenase in viable cells, producing

insoluble purple formazan crystals, which were subsequently dissolved in DMSO. After 4 h of incubation, the supernatant was carefully removed, leaving the formazan crystals at the bottom of the wells. Then, 150 μL of DMSO was added to each well, and the plates were gently shaken to ensure complete dissolution of the crystals. Absorbance was measured at 630 nm using a multifunctional microplate reader.

Intracellular monitoring of Cu-HOF-catalyzed CuAAC reaction. To evaluate the **Cu-HOF**-mediated copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction within cells, HeLa and L929 cells were co-incubated with 3-azido-7-hydroxycoumarin, phenylacetylene, and **Cu-HOF@HA**. The intracellular fluorescence intensity resulting from the CuAAC reaction was assessed using confocal laser scanning microscopy (CLSM). Briefly, cells were incubated with 3-azido-7-hydroxycoumarin (20 μM) and phenylacetylene (20 μM) for 4 h. After incubation, cells were washed twice with PBS to remove unreacted substrates. Subsequently, **Cu-HOF@HA** (20 μg mL^{-1}) was added, and the cells were further incubated for 9 h. Following the reaction period, cells were washed three times with PBS and stained with Mito Red for 15 min to label mitochondria. Finally, intracellular fluorescence signals were observed and recorded using CLSM.

Mitochondrial Membrane Potential Assay. To investigate the effect of nanoparticles on mitochondrial membrane potential, a mitochondrial membrane potential assay kit containing JC-1 was used (Sparkjade). After co-incubating for 24 h, the cell samples washed by PBS, and prepared according to the instructions of the kit. Finally, the changes in mitochondrial membrane potential after various treatments were detected by Cell imaging.

Cell Apoptosis of 1+2 and 1+2@Cu-HOF@HA Nanoparticles. To study the cell apoptosis process, Annexin V-FITC/PI Apoptosis Detection Kit was used. HeLa cells were treated with PBS, **Cu-HOF@HA** (10 μg mL^{-1}), 1+2 (10 μg mL^{-1}), and **1+2@Cu-HOF@HA** (10 μg mL^{-1}), respectively, and then the cells were incubated in 6-well plates overnight. After that, the cells were harvested and washed with PBS, and then resuspended with binding buffer (400 μL). At last, 5 μL of Annexin V-FITC and 5 μL of PI were utilized to stain the samples for 15 min and 5 min in the dark, respectively. The cell apoptosis process was monitored *via* a flow cytometer.

***In vivo* antitumor efficacy of 1+2@Cu-HOF@HA.** Female Balb/C nude mice (about 18 g) were purchased from the Hunan Silaikejingda Experimental Animal Co. Ltd. The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Guangxi Normal University (202506-005), and all procedures were performed in accordance with internationally accepted principles for the use and care of laboratory animals. The development of tumor model was achieved by subcutaneous injection of Hela cells into the right side of Balb/C nude mice. When the tumor volume reached up to 80-120 mm^3, 20 cancer-bearing mice were randomly divided into four groups, and treated with Control, **Cu-HOF@HA**（100 μL，4 $mg \cdot kg^{-1}$）, 1+2（100 μL，4 $mg \cdot kg^{-1}$）and **1+2@Cu-HOF@HA**（100 μL，4 $mg \cdot kg^{-1}$）, respectively. All groups of mice received twice drug injections on day 1 and day 7, respectively. The relative tumor volume was V/V_0 and the tumor volume was calculated by $V=4/3 \times \text{length} \times \text{width}\ 2/8$, where V_0 was the tumor volume before treatment.

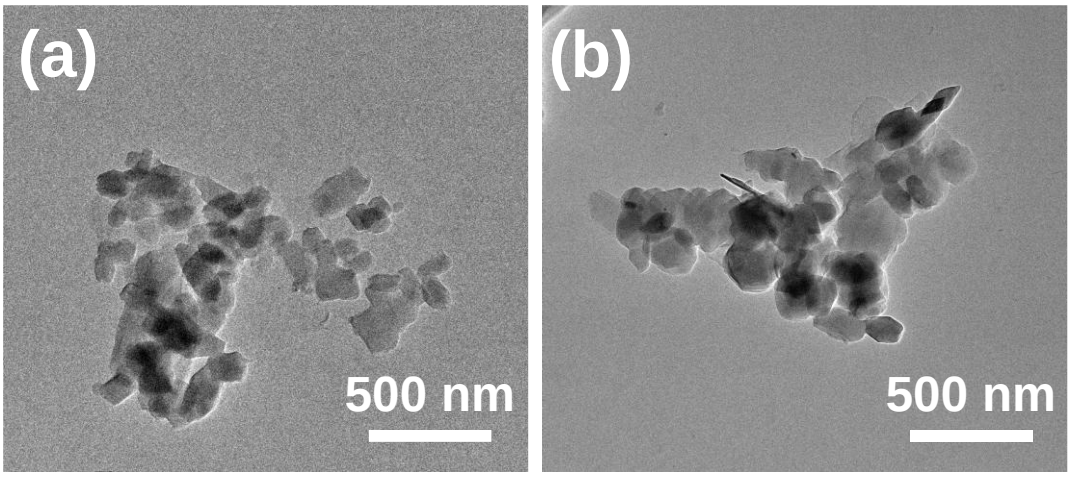

Fig. S1. TEM images of (a) **Cu-HOF**, (b) **1+2@Cu-HOF**.

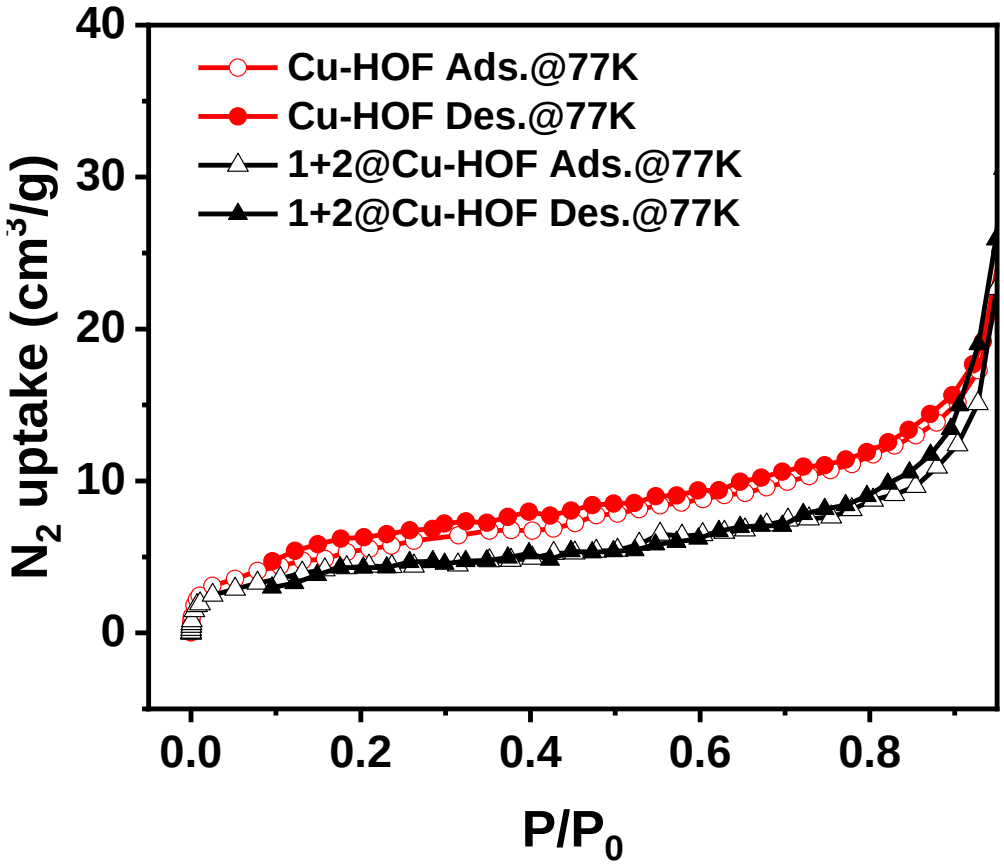

Fig. S2. The N_2 sorption isotherms and pore size distribution for **Cu-HOF** and **1+2@Cu-HOF**.

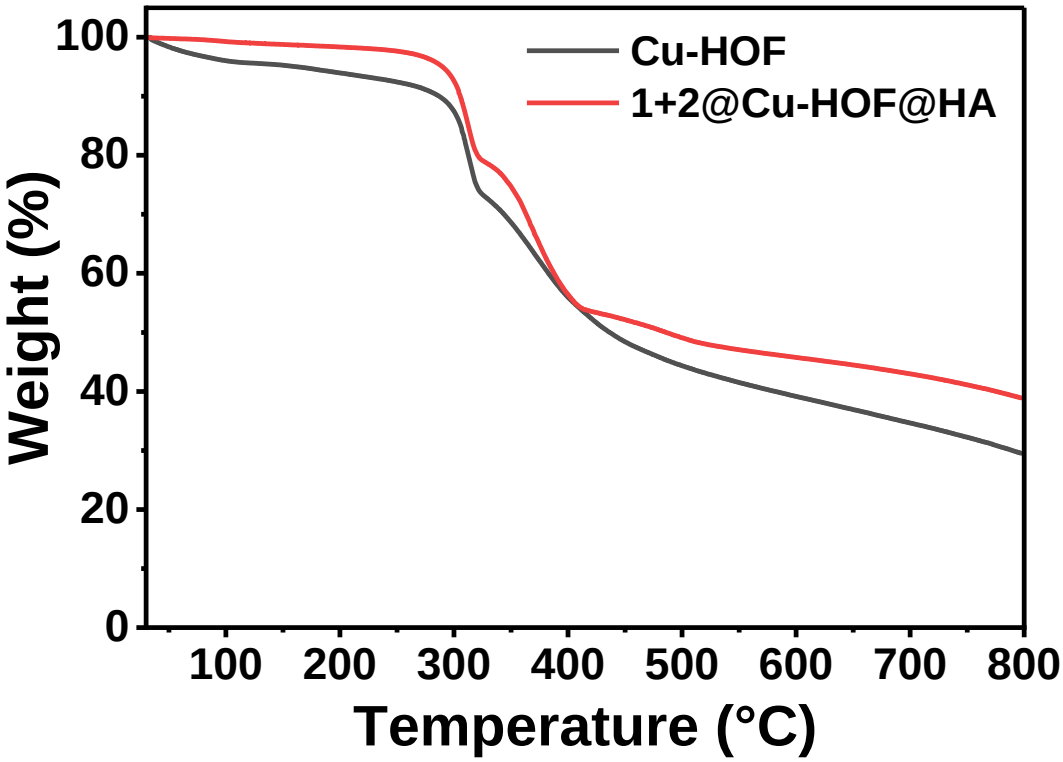

Fig. S3. The TGA curves for **Cu-HOF** and **1+2@Cu-HOF**, respectively.

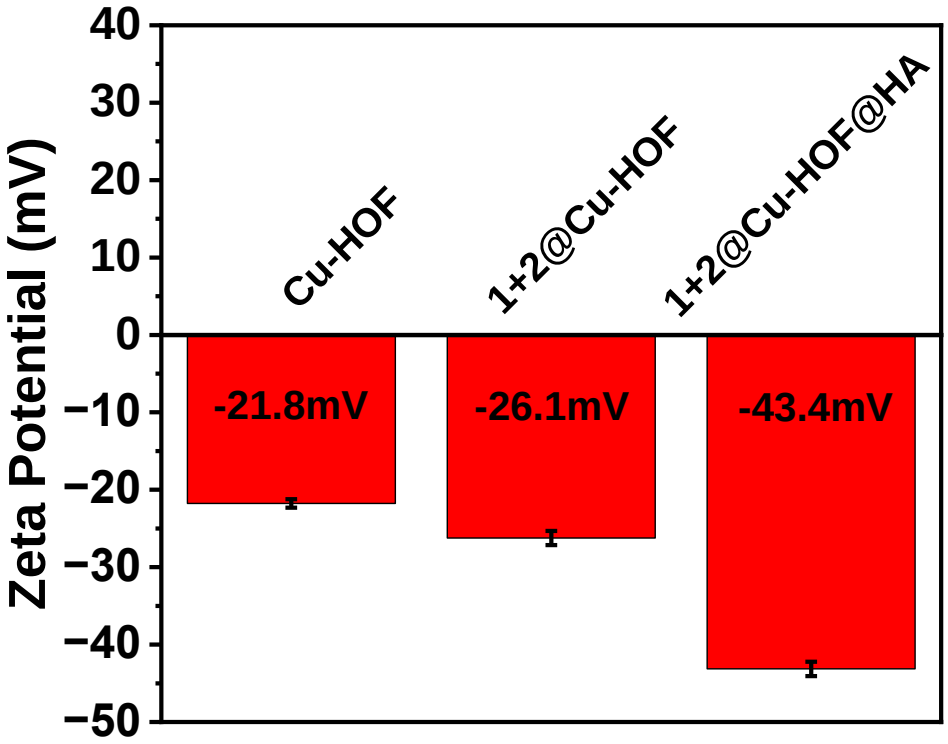

Fig. S4. Zeta potential of **Cu-HOF**, **1+2@Cu-HOF** and **1+2@Cu-HOF@HA**.

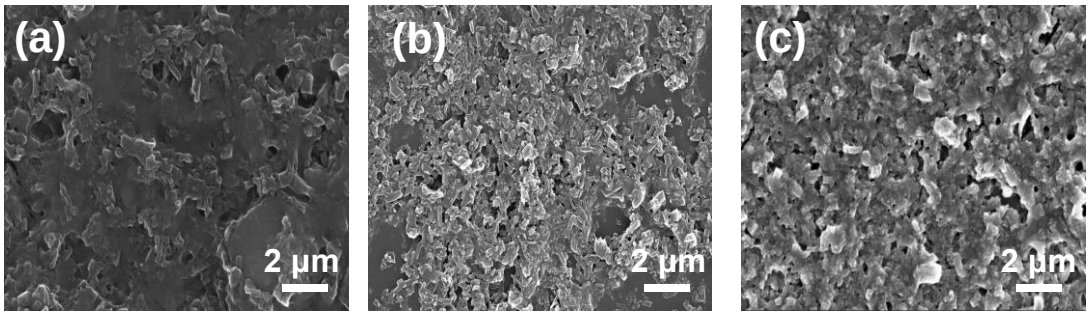

Fig. S5. (a)-(c) SEM images of **1+2@Cu-HOF@HA** dispersed in PBS (a) pH = 5.0, (b) pH = 7.4, and DMEM medium after 24 h, respectively.

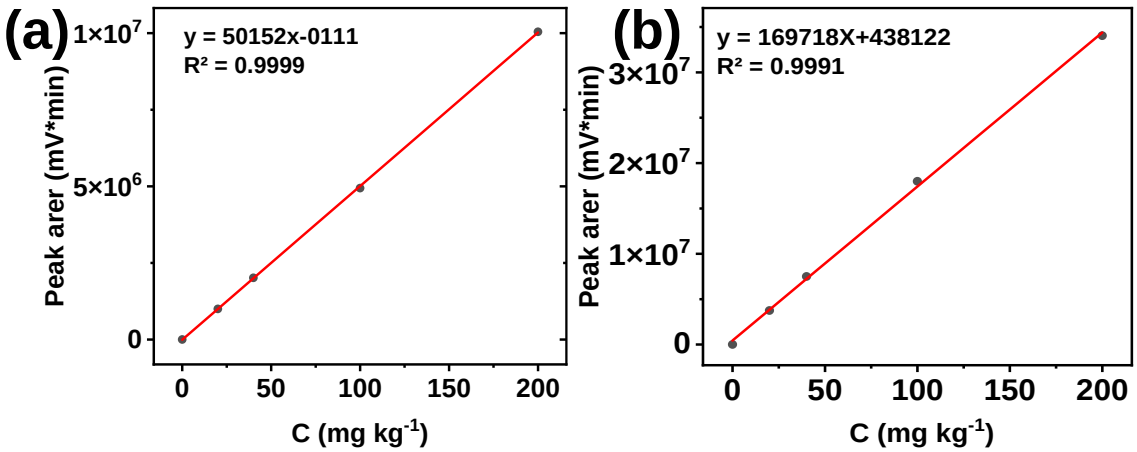

Fig. S6. Standard calibration curve of (a) precursor molecule 1 and (b) precursor molecule 2.

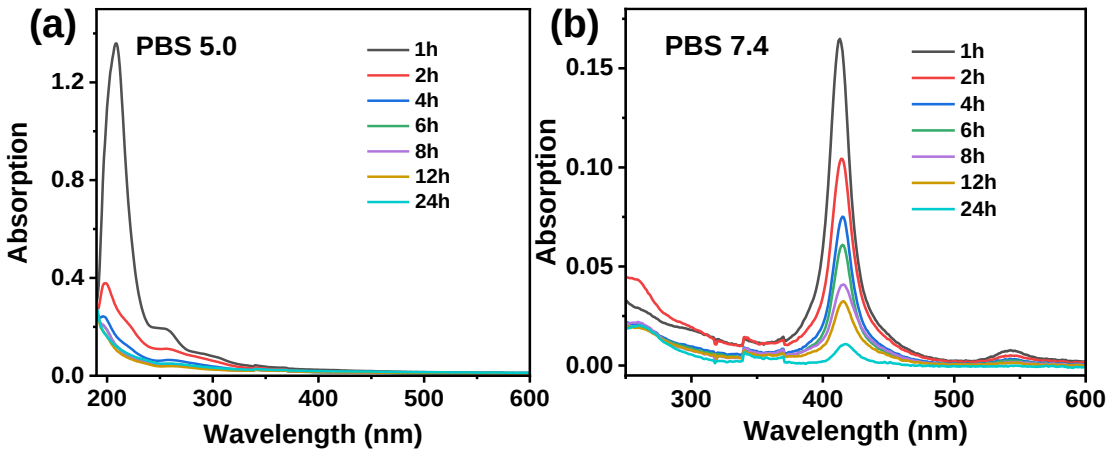

Fig. S7. Drug release profile in PBS solutions at different pH values.

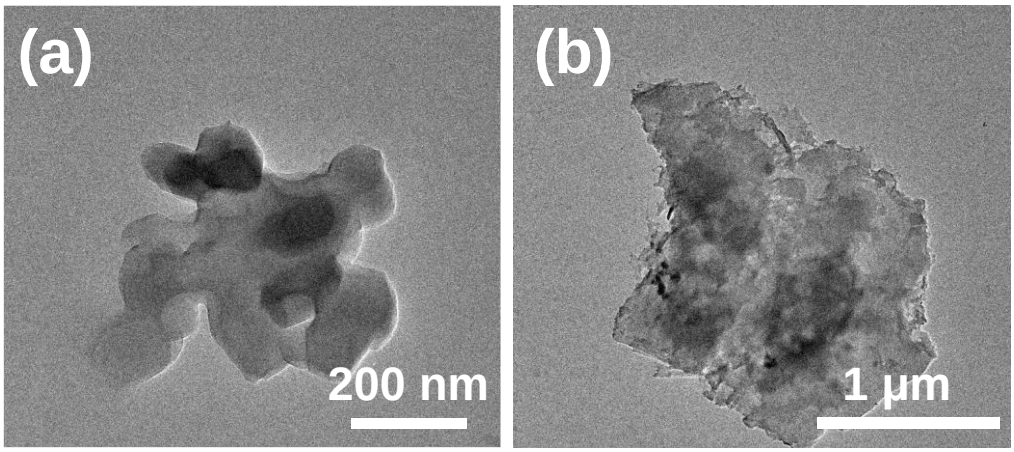

Fig. S8. Cu-HOF in the presence of GSH in H_2O, (b) **Cu-HOF** after co-incubation with HeLa cells for 24 h.

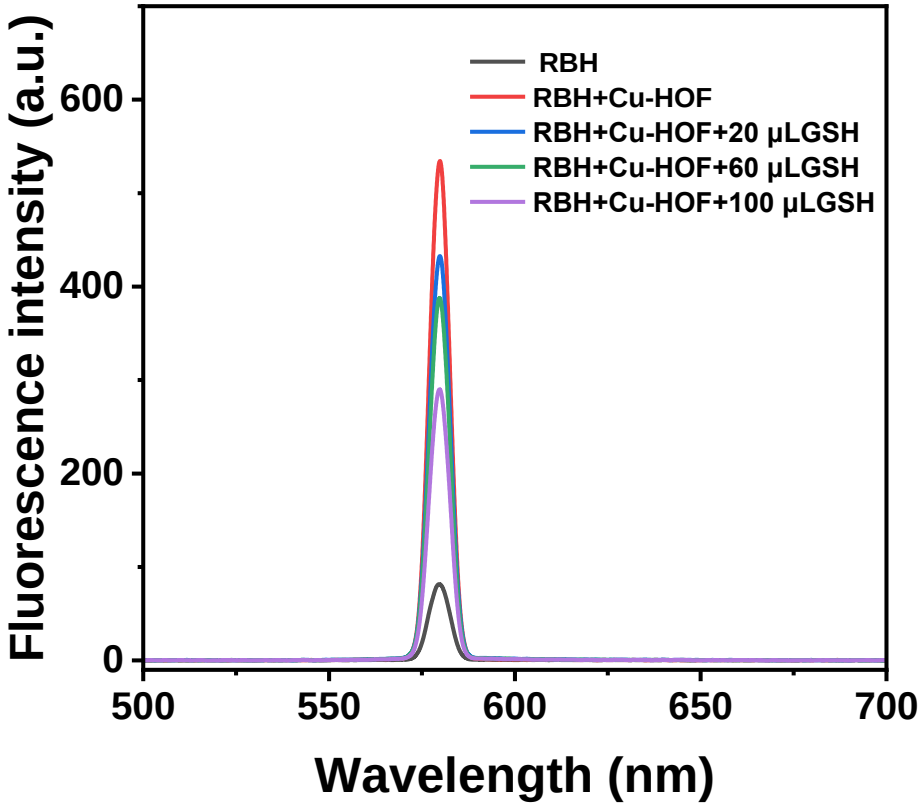

Fig. S9. Fluorescence spectra of simulating the GSH-catalyzed reduction of **Cu-HOF** in vitro.

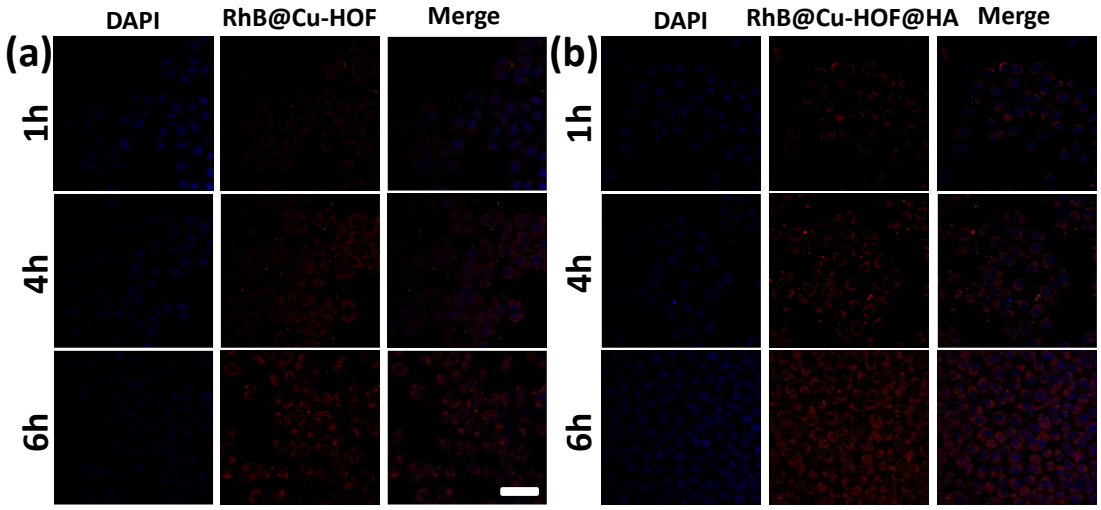

Fig. S10. CLSM images of HeLa cells incubated with RhB-modified (a) **Cu-HOF** and (b) **Cu-HOF@HA** for different times. Scale bars: 20 μm.

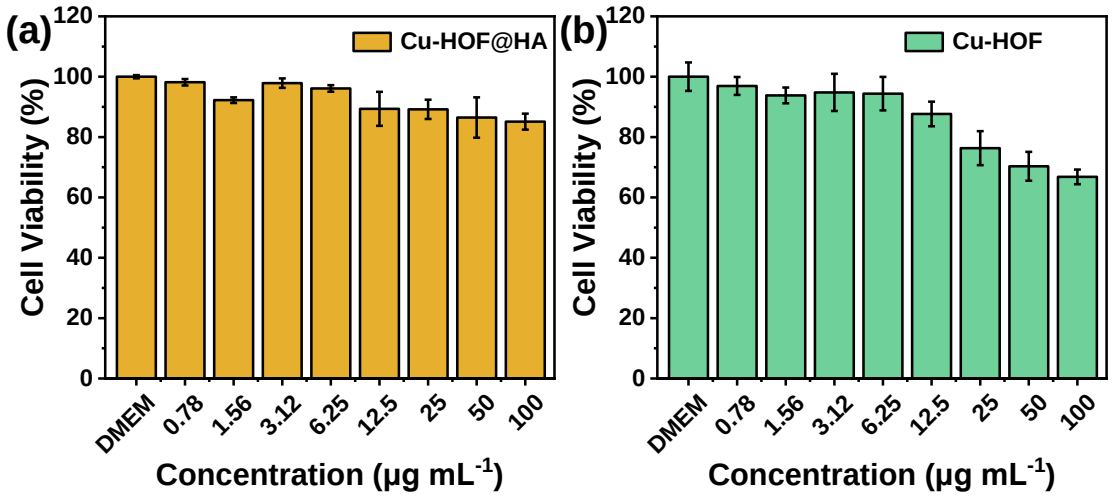

Fig. S11. Cell viability of HeLa cells treated with (a) **Cu-HOF@HA**, (b) **Cu-HOF**.

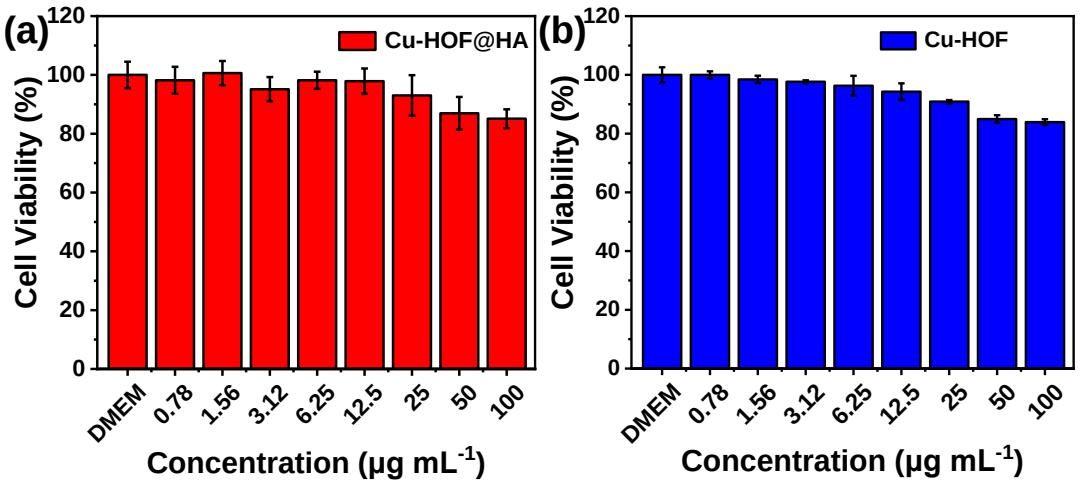

Fig. S12. Cell viability of L929 cells treated with (a) **Cu-HOF@HA**, (b) **Cu-HOF**.

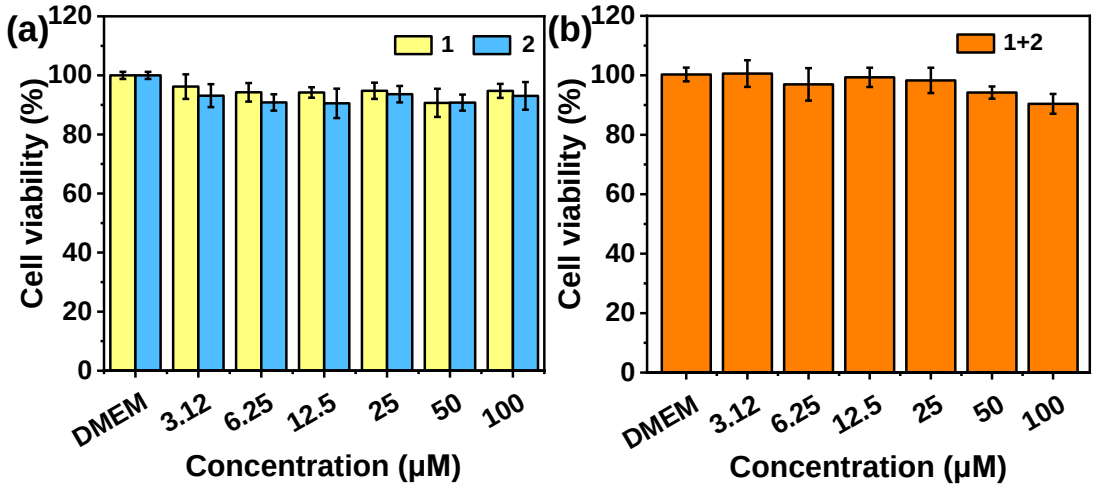

Fig. S13. (a) In vitro cell viability of prodrugs on 3T3 cells, (b) In vitro cell viability of 3T3 cells treated with 1 + 2.

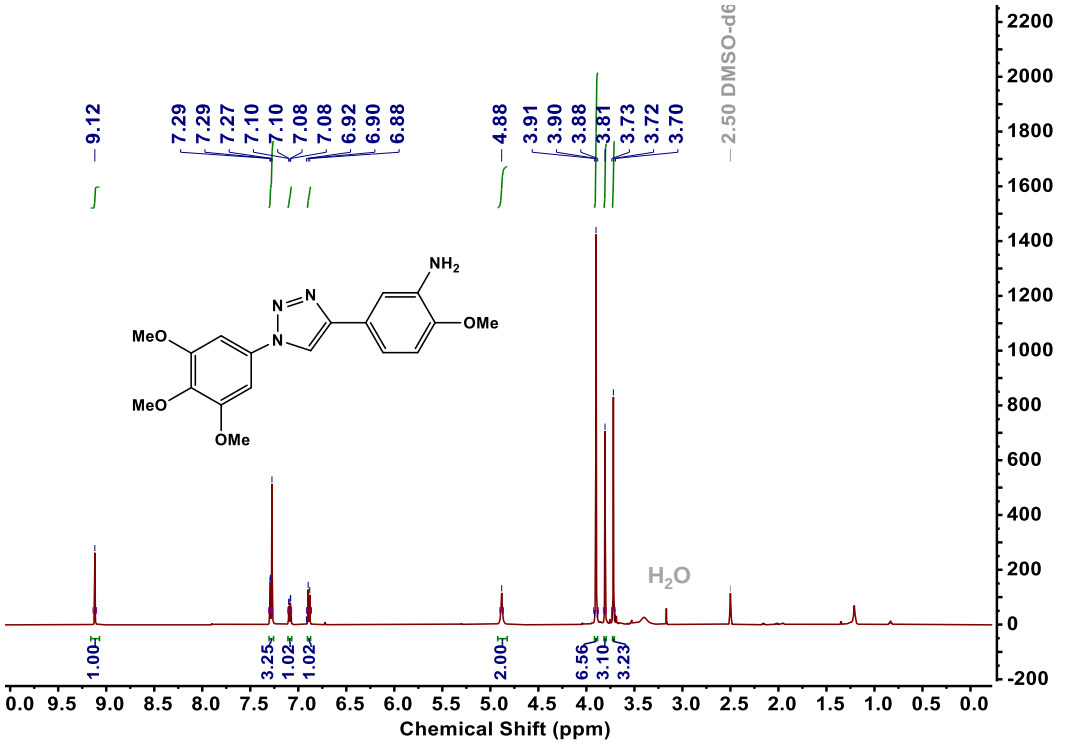

Fig. S14. ^{1}H NMR of drug 3 in DMSO- d_6.

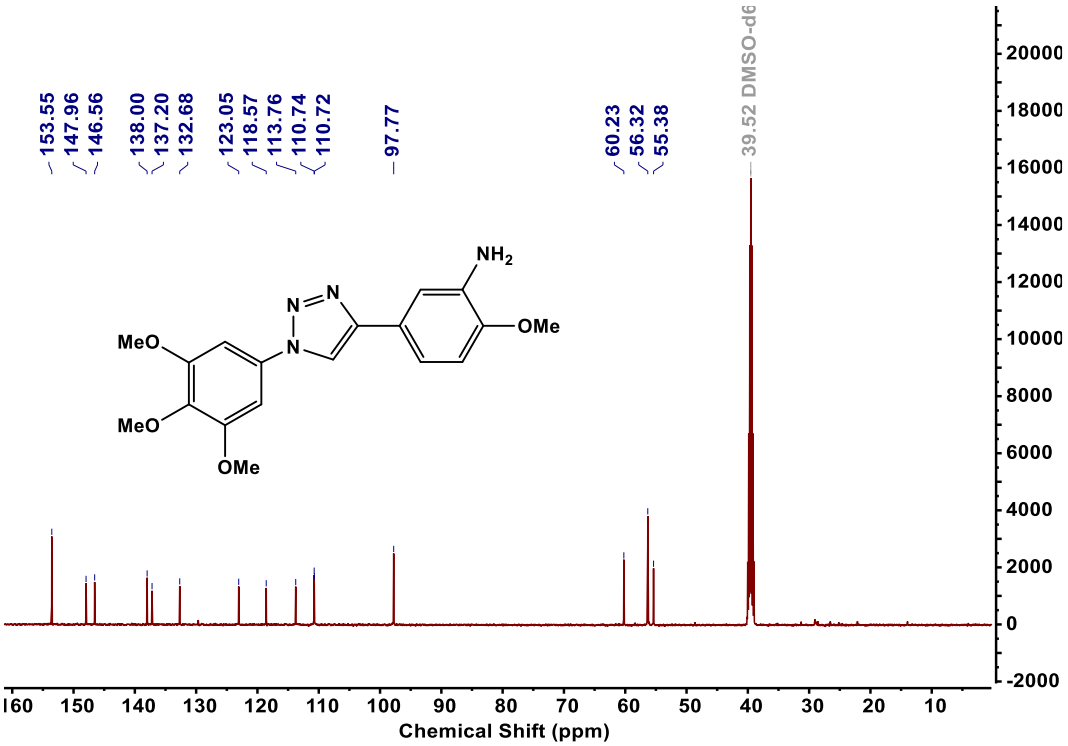

Fig. S15. ^{13}C NMR of drug 3 in DMSO- d_6.

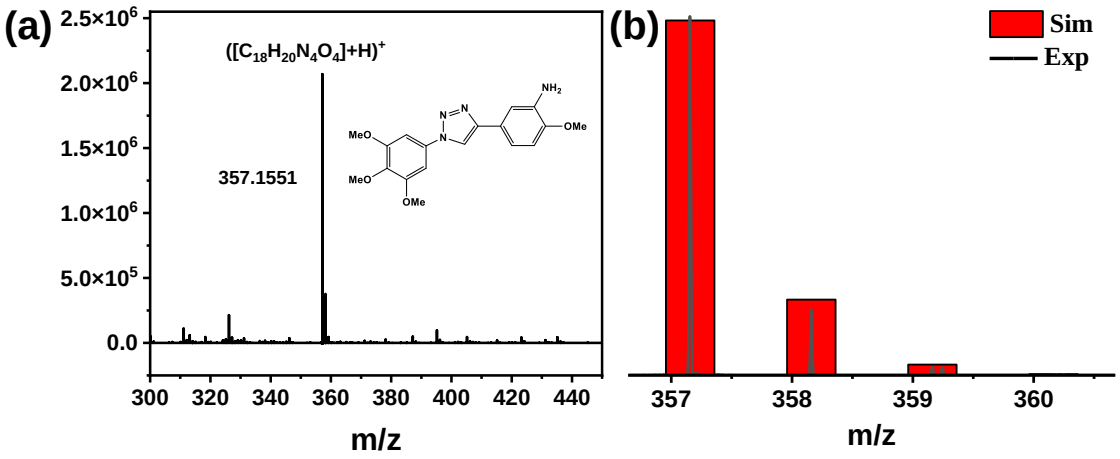

Fig. S16. HRMS spectrum of drug 3.

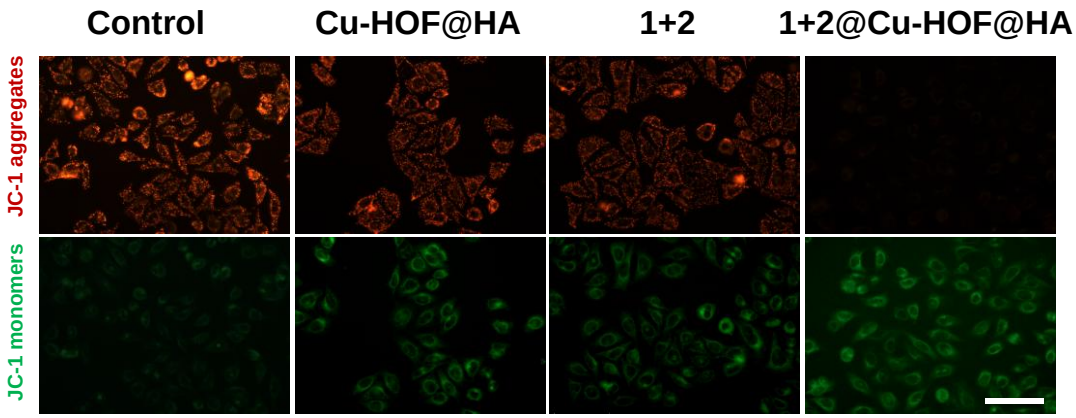

Fig. S17. Cell imaging of mitochondrial membrane potential. The scale bar is 100 mm.

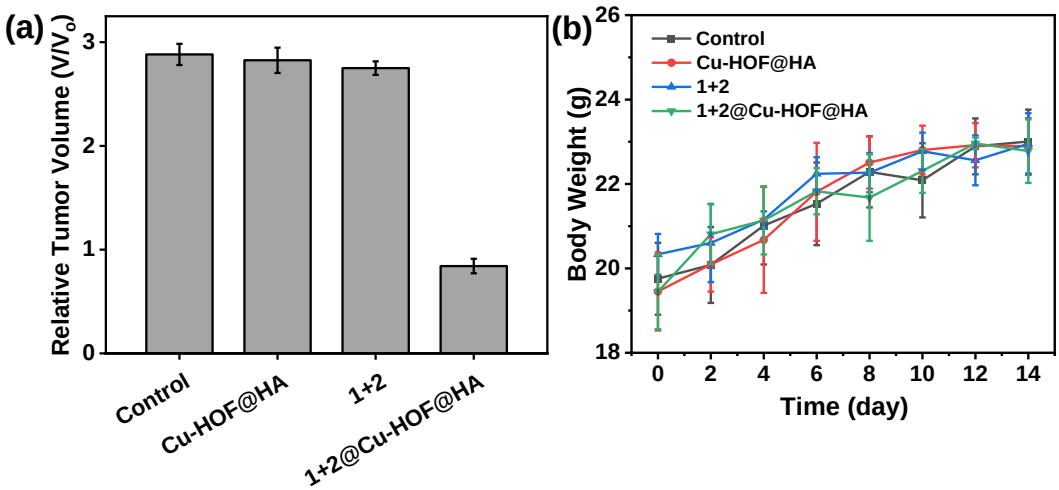

Fig. S18. (a) The changes in tumor volume on the 14th day after different treatments. (b) body weight changes by different treatments.

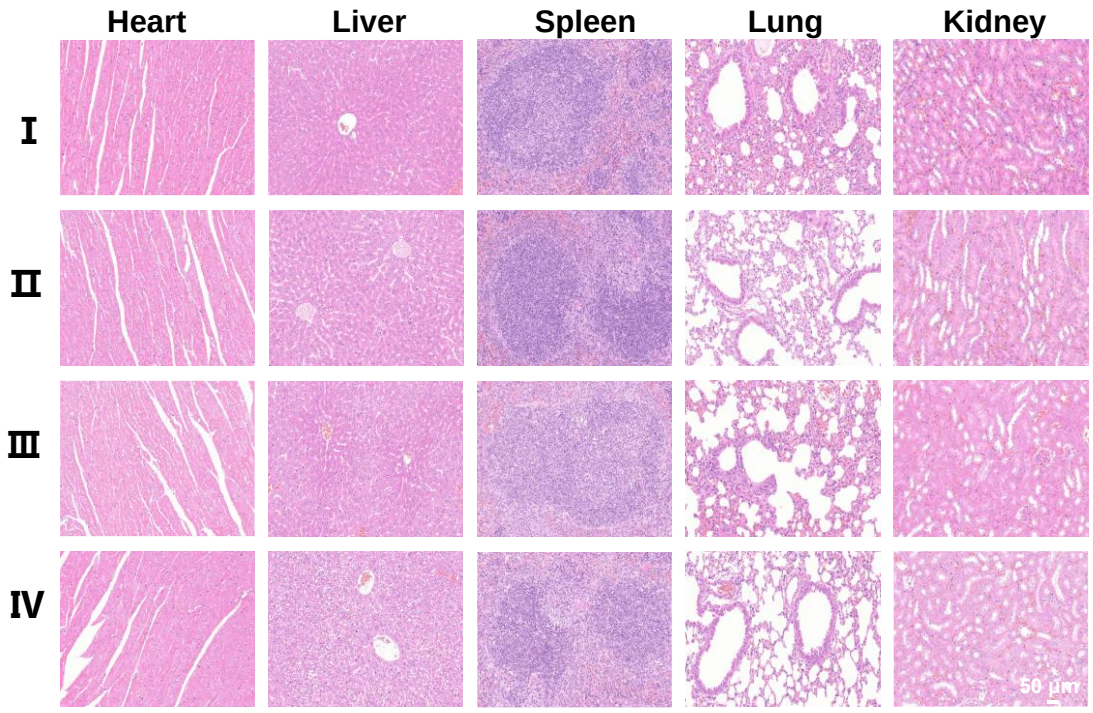

Fig. S19. Images of H&E stained slices of heart, liver, spleen, lung, kidney. Scale bars, 50 μm.