Cu-MOF-based targeted nanomedicine utilizing biorthogonally catalyzed chemotherapy and chemodynamic therapy with spatiotemporal orchestration to treat hepatocellular carcinoma

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Experimental Procedures

Materials and reagents

3-(4,5-dimethylthiazole-2)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. 1,3,5-Benzenetricarboxylic Acid (H_3BTC), $Cu(NO_3)_2 \cdot 3H_2O$, Glutathione (GSH), Dichlorobis (triphenylphosphine) palladium(II) ($Pd(PPh_3)_2Cl_2$), ethynyltrimethylsilane (TMS), cuprous iodide (CuI), 4-iodophenol, 5-aminobenzene-1,3-diol hydrochloride, (2S,3R,4R,5R,6R)-3-acetamido-6-(acetoxymethyl) tetrahydro-2H-pyran-2,4,5-triacetate (GalNAc-5OAc), 2-azidoethanol and trimethylsilyl trifluoromethanesulfonate (TMSOTf) were purchased from Shanghai Adamas Beta Chemical Reagent Co. Methylene Blue (MB) was purchased from Tianjin Hiens Biochemical Technology Co. 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Shanghai Anergy Chemical Co. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from Shanghai McLean Biochemical Technology Co. Calcein-AM/PI double staining kit was purchased from Nantong Biyuntian Company, China. Reactive oxygen detection kit (DCFH-DA) was purchased from Nantong Biyuntian Company, China. Dimethyl sulfoxide (DMSO), triethylamine (TEA), anhydrous ethanol and anhydrous methanol were purchased from Shanghai Sinopharm Chemical Reagent Co. Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS) were purchased from Shanghai Darthel Biotechnology Co. Mouse hepatocellular carcinoma cell line (Hepa 1-6) and Hepa 1-6-Luc cells transfected with fluorokinase were purchased from Ubigene Biosciences (Guangzhou, China). The water used for the experiments was Mill-Q secondary ultrapure water (18.2 $M\Omega \cdot cm^{-1}$). All other chemical reagents were of analytical grade and did not require further purification. All raw materials and solvents were obtained from commercial sources without further purification unless otherwise specified.

Instruments

Fourier Transform Infrared Spectrometer (IS50 FT-IR, Thermo, USA); Transmission Electron Microscope (HT7700, Hitachi, Japan); UV-Vis Spectrometer (TU-1901, PERSEE, Beijing); Enzyme Labeling Instrument (RT-6000, Rayto, USA); Laser Confocal Microscope (SP8, Leica, Germany) ; Digital pH meter (pH-3e, LeiCi, China);

Inductively Coupled Plasma Spectrometer (iCAD7400, Thermo, USA); X-ray Photoelectron Spectrometer (Escalab 250Xi, Thermo, USA). Live animal imaging system (IVIS Lumina III, USA); microplate reader (RT 6000, Rayto, USA).

Preparation of Cu-MOF-199:

The metal-organic framework (MOF) with the HKUST-1 structure was synthesized following the procedure reported in the literature. First, 6.3 mg of H_3BTC and 12.5 μL of triethylamine were added to 300 μL of deionized water to create a 0.1 M aqueous solution of H_3BTC triethylamine salt at room temperature. Next, 300 μL of this solution was combined with 30 mL of a 1:1 (v/v) mixture of ethanol and deionized water, resulting in solution A. Finally, 450 μL of a 0.1 M aqueous solution of $Cu(NO_3)•3H_2O$ was dripped into solution A. The reaction mixture was stirred vigorously for 30 minutes at room temperature. The resulting product was washed three times with ethanol to remove unreacted reagents. The final purified product was then placed in anhydrous ethanol and stored at room temperature.

Preparation of (1+2)@MOF：

The prodrug 5-azidobenzene-1,3-diol (**1**) (2 mg) and the metal-organic framework (MOF) (1 mg) were first dissolved in 1 mL of anhydrous ethanol and stirred for 12 hours at room temperature, avoiding exposure to light. The mixture was then centrifuged at 10,000 rpm for 5 minutes to isolate the **1**@MOF nanoparticles and collect the supernatant. The precipitate was washed twice with anhydrous ethanol. The amount of compound **1** in the supernatant was measured using UV-visible spectroscopy, which was used to calculate the loading of 1. Next, the prodrug 4-ethynylphenol (**2**) (1 mg) and the **1**@MOF nanoparticles (1 mg) were dissolved in 1 mL of anhydrous ethanol and stirred for 6 hours at room temperature, also avoiding light exposure. After the reaction, the mixture was centrifuged at 10,000 rpm for 5 minutes to isolate the (**1**+**2**)@MOF nanoparticles and collect the supernatant. The precipitate was again washed twice with anhydrous ethanol. The amount of compound **2** in the supernatant was detected using UV-visible spectroscopy, from which the loading of **2** was calculated.

Preparation of (1+2)@MOF-G

A solution of (**1**+**2**)@MOF (1 mg) and GalNAc-NH_2 (1 mg) was prepared by dissolving the compounds in 1 mL of anhydrous ethanol. The mixture was stirred at room temperature for 12 hours. After the reaction was completed, the resulting (**1**+**2**)@MOF-G nanoparticles were isolated by centrifugation at 10,000 rpm for 10 minutes.

Cu Release:

Dissolve 1 mg of MOF in 1 mL of a mixed solution of ethanol and water containing 10 mM GSH. After 24 hours, centrifuge the mixture and collect the supernatant. Determine the Cu content in the supernatant using an Inductively Coupled Plasma Emission Spectrometer (ICP).

Release of prodrugs:

Dissolve 1 mg of 1@MOF in 1 mL of a mixed solution of ethanol and water containing 10 mM GSH. After 2, 4, 6, 12, and 24 hours, centrifuge the mixtures and collect the supernatants. Measure the UV absorbance of the supernatants using a UV-Vis spectrophotometer.

Detection of ˙OH using Methylene Blue (MB):

Prepare 1 mL systems by adding 10 mM GSH, 100 mM H_2O_2, and 10 μg of MB probe to mixed solutions of ethanol and water containing 1 mg, 3 mg, and 5 mg of MOF, respectively. After 6 hours, centrifuge the mixtures and collect the supernatants. Additionally, prepare control groups with only the MB probe and with MB + H_2O_2. Measure the UV absorbance of all samples.

Detection of ˙OH using 3,3',5,5'-Tetramethylbenzidine (TMB):

Prepare 1 mL systems by adding 0.5 mM GSH, 2 mM GSH, 6 mM GSH, and 10 mM GSH to ethanol solutions containing 1 mg of MOF, along with 100 mM H_2O_2 and 0.8 mM TMB. After 6 hours, centrifuge the mixtures and collect the supernatants. Additionally, prepare control groups with only the TMB probe and with TMB + H_2O_2. Measure the UV absorbance of all samples.

GSH depletion was determined by DTNB:

1. Preparation of DTNB storage solution: Dissolve 0.01 mol/L DTNB in 0.05 mol/L phosphate buffer (pH=7.0) to form DTNB storage solution.

2. Preparation of DTNB analytical solution: The DTNB analytical solution was prepared by diluting the DTNB stock solution 100 times with 0.5 mol/L, pH = 8.0 Tris-HCl buffer solution, and stored in the dark, ready for current use.

3. Procedure: (1) Take 0.1 mL of a 10 mM GSH standard solution and add 300 μL of a 0.15 mol/L NaOH solution. Then, add 1 mL of a 3% formaldehyde solution and allow the mixture to react for 2 minutes at 25°C. After the reaction, add 120 μL of the DTNB analytical solution to the reaction mixture, and subsequently measure the UV-Vis absorption curve. (2) Dissolve 1 mg, 2 mg, and 5 mg of MOF in the 10 mM GSH solution. After 2 hours and then again after 6 hours of reaction time, take 100 μL of the reaction solution from each time point. Add this to 300 μL of a 0.15 mol/L NaOH solution. Next, incorporate 1 mL of a 3% formaldehyde solution and allow the mixture to react for 2 minutes at 25°C. Finally, add 120 μL of the DTNB analytical solution and measure the UV-Vis absorption curve.

Synthesize compound 3:

5 mg of prodrug 1 (5-azidobenzene-1,3-diol) and 10 mg of prodrug 2 (4-ethynylphenol) were dissolved in 2 mL of methanol. This solution was then added to a 6 mL mixture of ethanol and deionized water in a 1:1 (v/v) ratio, containing 10 mM glutathione (GSH). Additionally, 10 mg of metal-organic framework (MOF) nanoparticles were included in the mixture. The reaction was stirred at room temperature for 12 hours. After the reaction was complete, the supernatant was collected by centrifugation, and the product was analyzed using high-resolution mass spectrometry (HRMS).

Cell Culture:

Hepa1-6 cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin) and maintained at 37 °C in a 5% CO_2 humidified incubator.

Cellular uptake:

1. RhB@MOF-G was first prepared: RhB@MOF-G was prepared by mixing 1 mg of MOF-G nanoparticles with 500 μL of a 1 mg/mL Rhodamine B (RhB) solution in 1 mL of ethanol. The mixture was stirred in the dark for 12 hours to protect it from light. After stirring, RhB@MOF-G was isolated by centrifugation at 14,000 rpm for 10

minutes. The resulting product was washed three times with anhydrous ethanol and then set aside.

2. Hepa1-6 cells and AML-12 cells were inoculated in a confocal culture dish and cultured for 24 hours. The two types of cells were then divided into two groups and incubated with RhB@MOF (200 μg/mL) and RhB@MOF-G (200 μg/mL) for 3 hours. After this incubation period, the cells were analyzed using laser confocal microscopy.

MTT assay:

1. Hepa1-6 cells were inoculated into 96-well plates and incubated for 24 hours. After this incubation period, the contents of the wells were removed, and various concentrations of compounds 1, 2, and 3 (15, 25, 35, 45, and 60 μg/mL) were added. The plates were then incubated for an additional 24 hours. Following this, 150 μL of MTT solution (0.5 mg/mL) was added to each well and incubated for 4 hours. After incubation, the MTT solution was aspirated, and 150 μL of DMSO was added to each well. The absorbance was measured at 490 nm using a spectrophotometer.

2. Hepa1-6 cells were inoculated into 96-well plates and incubated for 24 hours. The cells were divided into five groups: PBS, 1+2, MOF-G, (1+2)+MOF-G, and (1+2)@MOF-G. The materials for each group were removed from the 96-well plates after being incubated for 24 hours with varying concentrations of MOF: 50, 100, 150, and 200 μg/mL, respectively. Following the incubation, 150 μL of MTT solution (0.5 mg/mL) was added and incubated for an additional 4 hours. After incubation, the MTT solution was aspirated, and 150 μL of DMSO was added. The absorbance was then measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) meter.

Detects the level of intracellular ROS production:

Hepa1-6 cells were plated in confocal dishes and cultured overnight in five groups: PBS, GalNAc, MOF, and MOF-G. The cells were treated with PBS, GalNAc, MOF (200 μg/mL), and MOF-G (200 μg/mL) and incubated for 12 hours. After this, the Hepa1-6 cells were exposed to the DCFH-DA (1 μg/mL) probe for 20 minutes. Finally, the cells were washed three times with PBS and analyzed using laser confocal microscopy.

Live/dead cell staining analysis:

Hepa1-6 cells were inoculated into confocal dishes and cultured overnight, divided into five treatment groups: PBS, 1+2, MOF-G, (1+2)+MOF-G, and (1+2)@MOF-G. The cells were treated with the following: PBS, 1+2 (where 1 = 54 μg/mL and 2 = 18 μg/mL), MOF-G (200 μg/mL), (1+2)+MOF-G (1 = 54 μg/mL, 2 = 18 μg/mL, and MOF-G = 200 μg/mL), and (1+2)@MOF-G (200 μg/mL). After incubation for 24 hours, Calcein-AM (1 μg/mL) and propidium iodide (PI) (1 μg/mL) probes were added to the confocal dishes and incubated for an additional 20 minutes. Finally, the dishes were washed three times with PBS and analyzed using laser confocal microscopy.

Cell proliferation assay:

Hepa1-6 cells were inoculated and cultured in six-well plates, divided into five groups: PBS, 1+2, MOF-G, (1+2)+MOF-G, and (1+2)@MOF-G. The cells were then incubated with the following solutions: PBS, 1+2 (where 1 = 54 μg/mL and 2 = 18 μg/mL), MOF-G (200 μg/mL), (1+2)+MOF-G (1 = 54 μg/mL, 2 = 18 μg/mL, and MOF-G = 200 μg/mL), and (1+2)@MOF-G (200 μg/mL). After a 24-hour incubation period, the media were removed, and the cells were washed with PBS. They were then fixed with paraformaldehyde for 15 minutes, followed by incubation with crystal violet dye for 25 minutes, protected from light. After incubation, the excess dye was aspirated, and the cells were washed with PBS to remove any leftover dye.

Establishment of a transplanted tumor model for hepatocellular carcinoma in situ:

Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU2023056). All animal experiments were conducted and obeyed the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China. C57 mice (4-5 weeks, male) were used as animal models and fed for approximately one week to reach a body weight of approximately 18 g. Hepa 1-6-Luc cells were digested with trypsin and then washed three times with PBS. Approximately every 1×10^7 Hepa 1-6-Luc cells were dispersed in 50 μL of serum-free DMEM

medium, and the cell suspension was injected into the mouse liver to form an in situ transplantation tumor model.

In vivo fluorescence imaging:

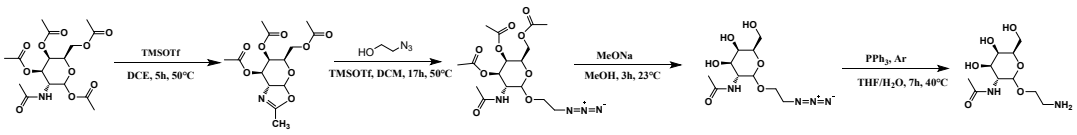
1. First, IR808@MOF-G was prepared by mixing 10 mg of MOF-G nanoparticles with 1 mg of IR808 in a 10 mL ethanol solution. The mixture was stirred for 12 hours in a dark place, away from light. Afterward, IR808@MOF-G was isolated by centrifugation at 14,000 rpm for 10 minutes. The resulting particle was then washed three times with anhydrous ethanol and set aside for further use.

2. IR808@MOF (4 mg/mL, 50 μL) and IR808@MOF-G (4 mg/mL, 50 μL) were injected into C57 mice via the tail vein. The mice were then subjected to fluorescent imaging using an in vivo imaging system at 2, 8, 12, 24, 36, and 48 hours post-injection.

In vivo antitumor therapy experiments:

After 7 days, the mice were randomly divided into five groups: PBS, 1+2, MOF-G, (1+2)@MOF, and (1+2)@MOF-G. Each group was injected with nanoparticles (10 mg/kg) via the tail vein. Tumor size was monitored using bioimaging at different time points (days 0, 7, and 14). On day 14, the mice were euthanized, and blood samples were collected for routine hematological and biochemical analysis. Major organs (heart, spleen, lung, and kidney) as well as liver tissues were collected for H&E staining. The body weight of the mice was recorded every other day throughout the experiment.

Synthesis of GalNAc-NH_2.



1.17 g of GalNAc-5OAc (3 mmol, 1 eq) was dissolved in 1,2-dichloroethane (DCE, 30 mL) under 0 ℃. Then 667 μL of TMSOTf (3.9 mmol, 1.3 eq) was added dropwise. After stirring at 0 ℃ for 10 min, the reaction system was moved to a 50 ℃ oil bath and refluxed for 5 h. Then the reaction mixture was diluted with DCE and quenched with TEA. The mixture was washed with saturated $NaHCO_3$ and saturated NaCl solution. Finally, the organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The product (named as product A, yield 80%) of this step was used directly without further purification.

Product A (2.4 mmol, 1.2 eq) and 140 μL of 2-azidoethanol (2 mmol, 1 eq) was dissolved in DCM (18 mL) under 0 ℃. And 103 μL of TMSOTf (0.6 mmol, 0.3 eq) was added dropwise. After stirring at 0 ℃ for 10 min, the reaction system was moved to a 50 ℃ oil bath and refluxed for 17 h. Then the reaction system was quenched with TEA and purified by silica gel chromatography with CH_2Cl_2/CH_3OH (v/v, 20:1) as eluent, affording GalNAc-5OAc-N_3 as a pale-yellow solid.

416 mg of GalNAc-5OAc- N_3 (1 mmol, 1.0 eq) and 167 mg of MeONa (0.31 mmol, 0.31 eq) was dissolved in 10 mL of MeOH (pH 9). The system was allowed to stir at 23 ℃ for 3 h. Then ion-exchange resin was added into the solution until the pH 5-6. Finally, the resin was filtered, and the filtrate was dried to obtain GalNAc- N_3 as a pale-yellow solid.

58 mg of GalNAc- N_3 (0.2 mmol) and 144.26 mg of PPh_3 was dissolved in 2.5 mL of THF. Then 250 μL of H_2O was added. The reaction system was moved to a 40 ℃ oil bath and refluxed for 7 h under argon atmosphere. After dried, the product was washed with HCl (0.5 mol/L) and CH_2Cl_2 solution. Finally, the water phase was evaporated under reduced pressure to obtain GalNAc-NH_2. The GalNAc-NH_2 was used directly without further purification.

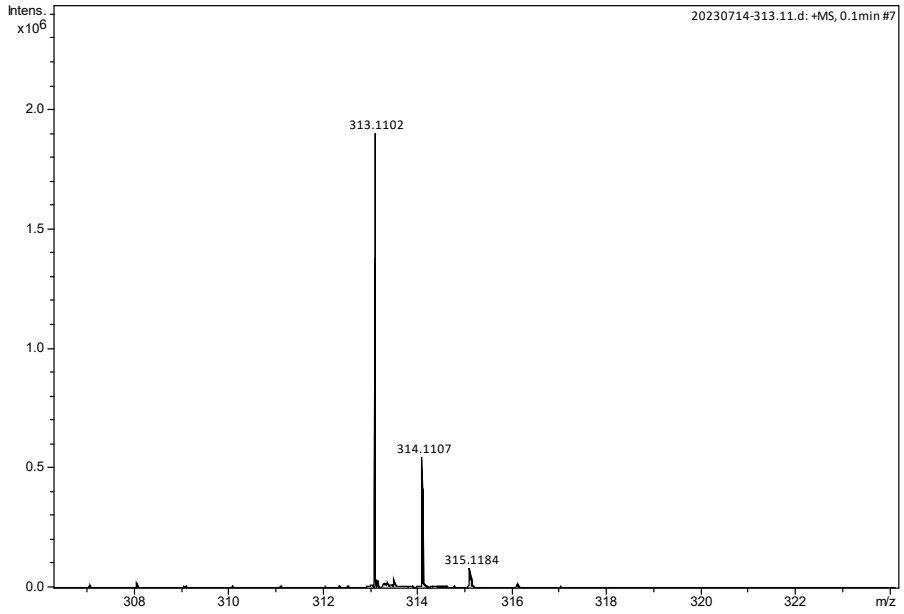


Figure S1. The HRMS spectrum of GalNAc-N_3.

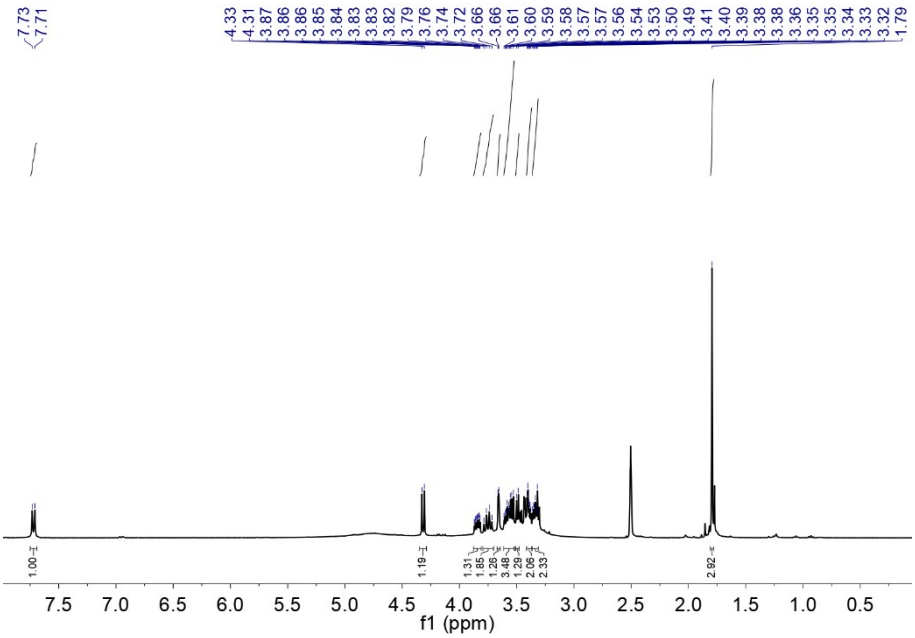
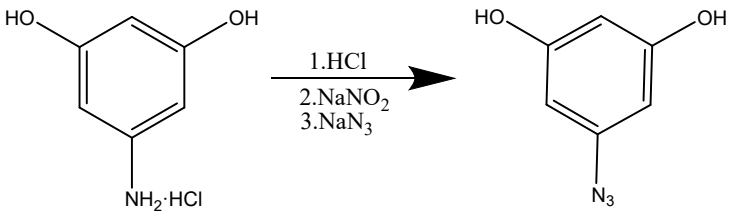


Figure S2. The ^{1}HNMR spectrum of GalNAc-N_3.

Synthesis of Chemical Substrate Molecules

Synthesis of 5-azidobenzene-1,3-diol (1).



5 g 5-Aminoresorcinol hydrochloride was added in 12.5 mL distilled water and 12.5 mL conc. HCl under 0 °C. 1.97 g $NaNO_2$ (28 mmol) in 12.5 mL H_2O was added to the solution slowly (> 5 min). 10 min later, 2.13g NaN_3 (33 mmol) in 12.5 mL H_2O was added to the solution and the solution was stirring for 40 min under 0 °C. The solution extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with brine (1 x 20 mL), dried over anhydrous $MgSO_4$, and concentrated under vacuum. The crude product was chromatographed with Hexane/EtOAc as eluant, to afford the light yellow crystal, **1**.[2]

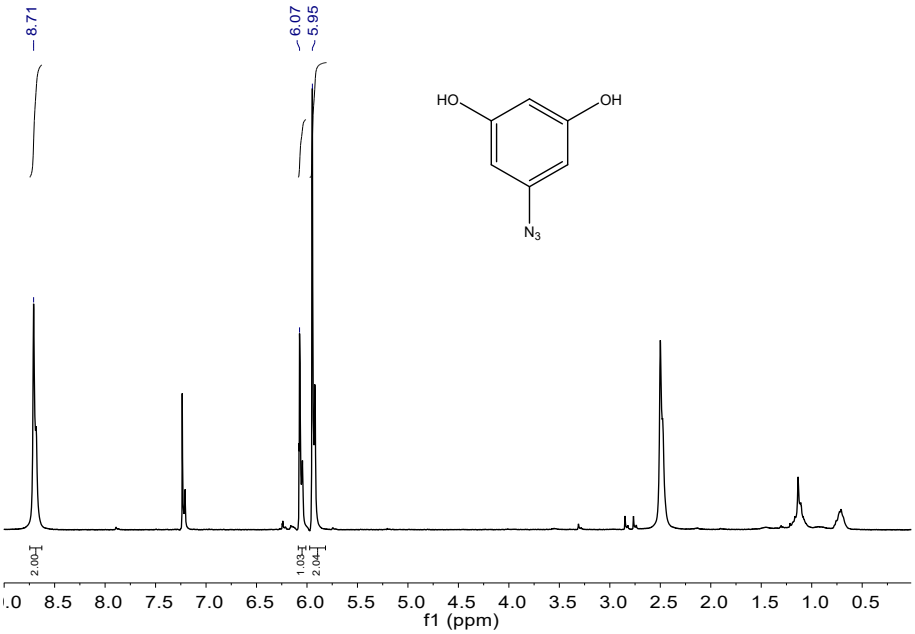
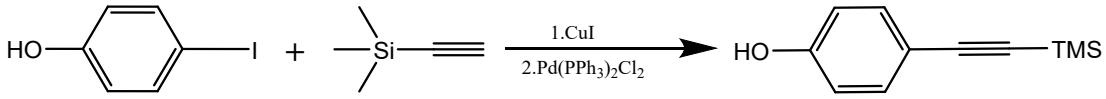


Figure S3. [1]HNMR spectrum of 5-azidobenzene-1, 3-diol, prodrug **1**.

Synthesis of 4-ethynylphenol (2)

4-((trimethylsilyl)ethynyl) phenol



Ethynyltrimethylsilane (0.950 mL, 6.72 mmol) was added to a solution of 4-iodophenol (1.02 g, 4.64 mmol), Pd (PPh3)2 Cl2 (97.2 mg, 0.140 mmol) and CuI (26.7 mg, 0.140 mmol) in Et3N (15 mL) and the mixture was refluxed at 80 °C for 3h under nitrogen. The solution was then cooled to room temperature, filtered, concentrated in vacuo and the crude product was chromatographed with Hexane/EtOAc as eluant, to obtain the precursor of compound (**2**) thetrimethylsilylethynylphenol (880 mg, 4.62 mmol, quantitative) as the brown oil.

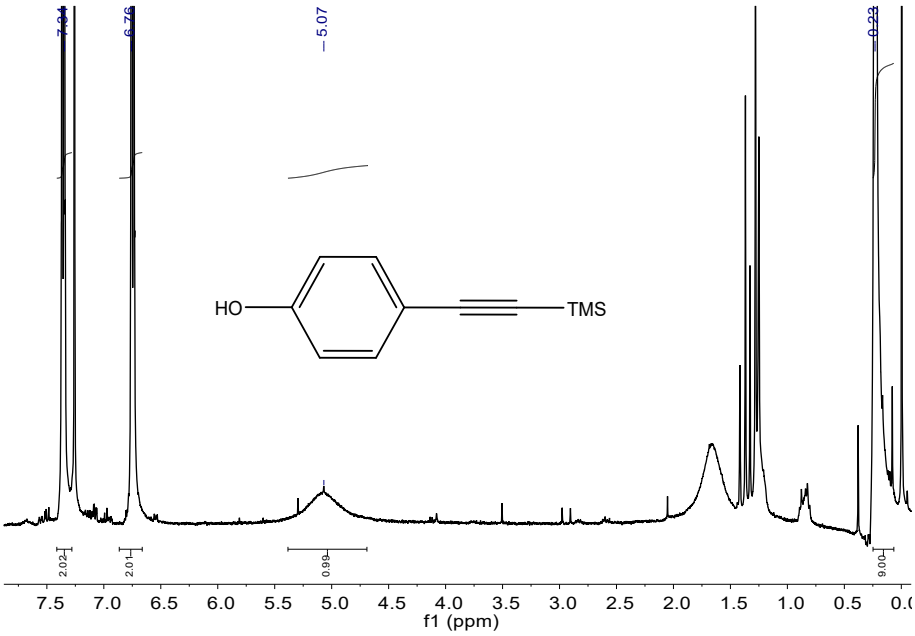
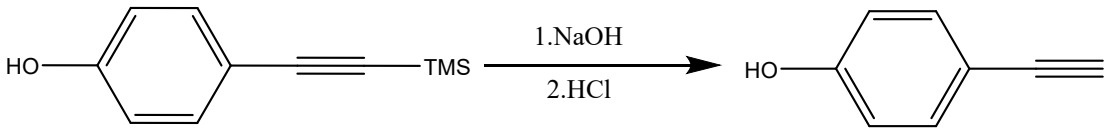


Figure S4. [1]HNMR spectrum of 4-((trimethylsilyl)ethynyl)phenol.

4-ethynylphenol (2)



Aqueous NaOH (5 M, 2 mL) was added to a solution of 4-((trimethylsilyl) ethynyl) phenol (520 mg, 2.74 mmol) in MeOH (10 mL) and the mixture was stirred at room temperature under nitrogen for 3 h, then neutralized with conc. HCl and extracted with DCM (3 x 20 mL). The combined organic layers were washed with brine (1 x 20 mL), dried over anhydrous $MgSO_4$, concentrated in vacuo and the crude product was chromatographed with Hexane/EtOAc as eluant, to afford the ethynylphenol (187 mg, 1.58 mmol, 58 %) as a dark red solid.[3]

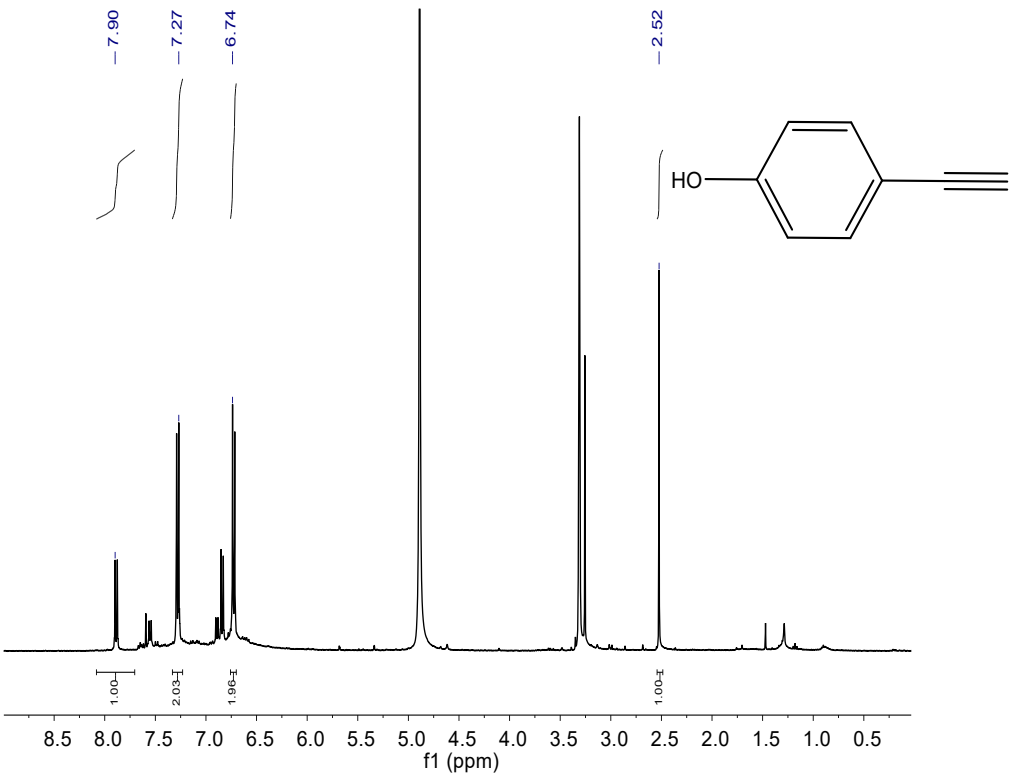


Figure S5. ^{1}HNMR spectrum of 4-ethynylphenol, **2**

Supporting figures

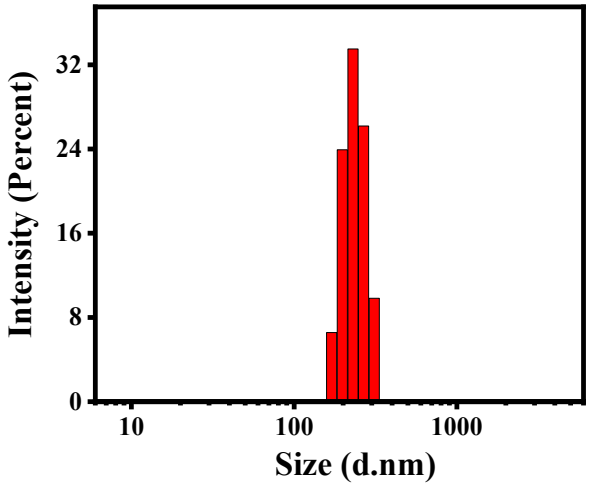


Figure S6. DLS measured size distribution of MOF.

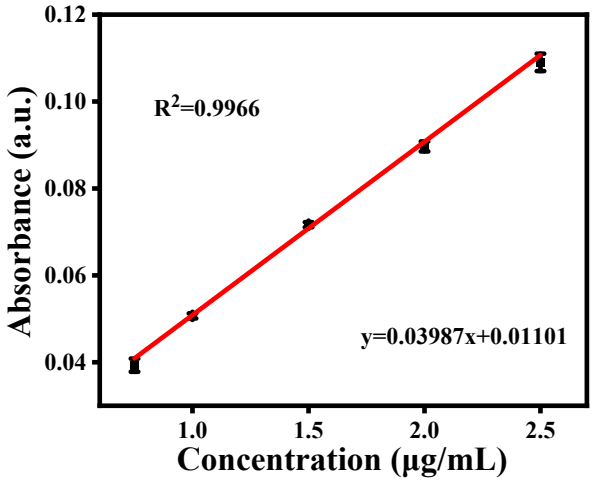


Figure S7. Linear relationships between the absorbance intensity of prodrug **1** and prodrug **1** concentration.

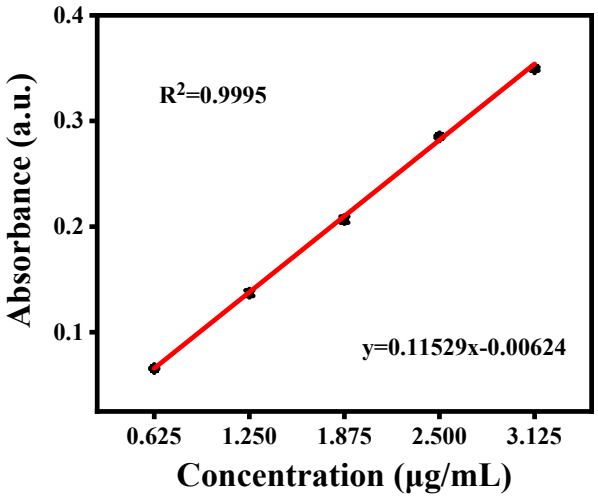


Figure S8. Linear relationships between the absorbance intensity of prodrug **2** and prodrug **2** concentration.

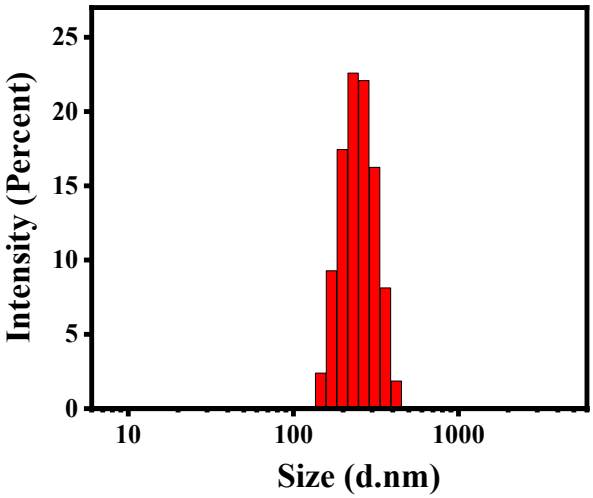


Figure S9. DLS measured size distribution of (**1**+**2**)@MOF.

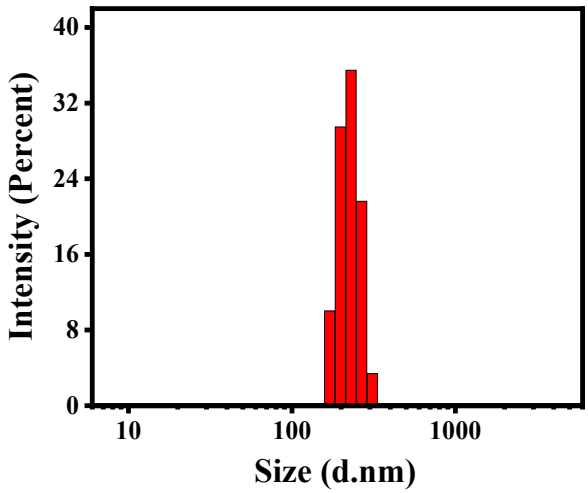


Figure S10. DLS measured size distribution of (**1**+**2**)@MOF-G.

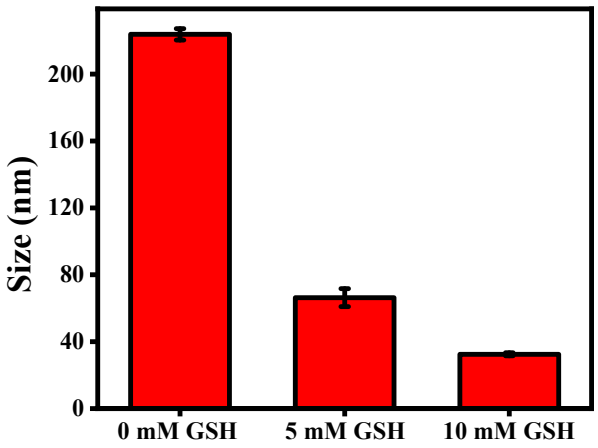


Figure S11. DLS size of MOF responded to GSH with different concentrations.

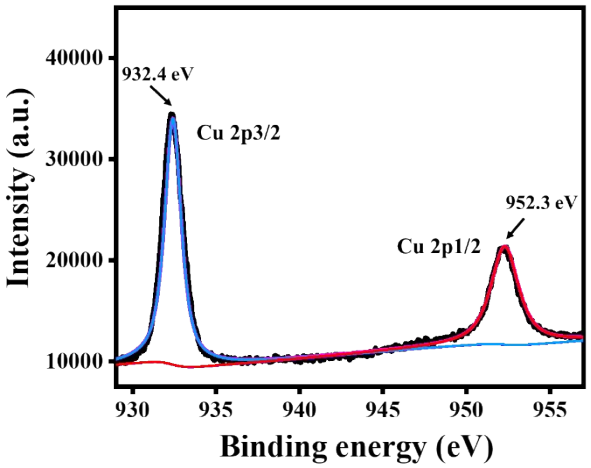


Figure S12. XPS spectra for Cu 2p regions of precipitate from the MOF solution treated with GSH.

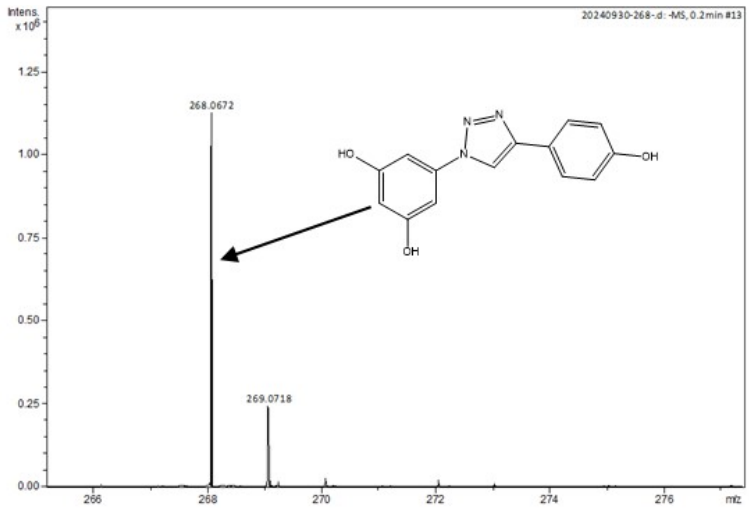


Figure S13. HRMS analysis of the reaction system containing MOF, GSH, and prodrugs **1** and **2** confirmed the presence of click coupling product **3**.

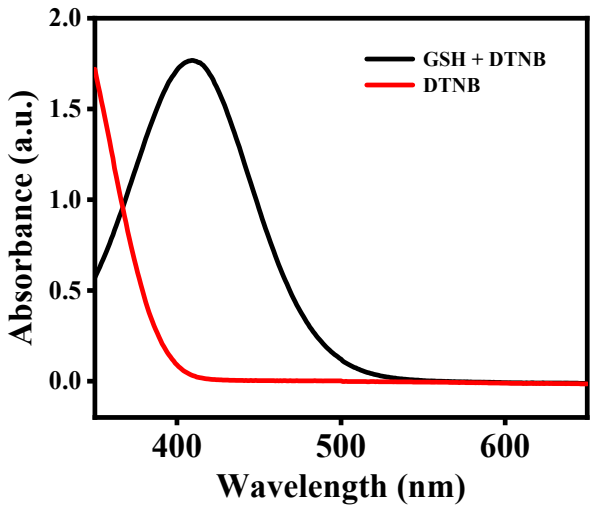


Figure S14. The UV-Vis spectra of DTNB with or without treatment of GSH.

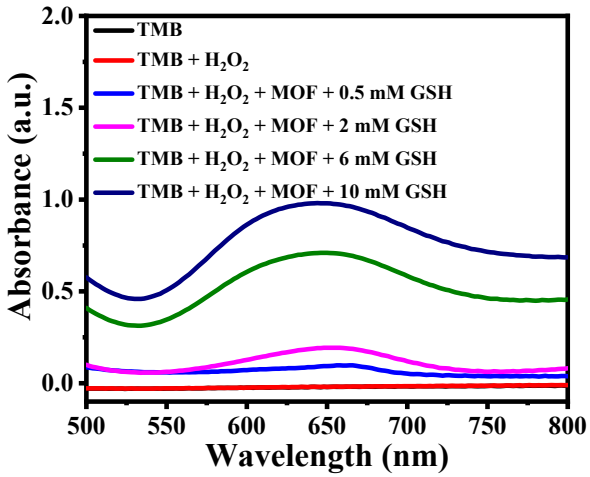


Figure S15. UV-Vis spectra of TMB treated with 100 mM H_2O_2, 1 mg MOF and different concentration of GSH.

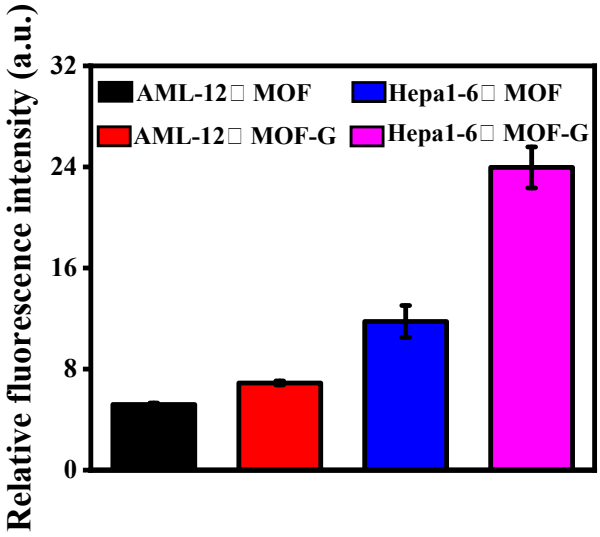


Figure S16. Quantitative analysis of the mean fluorescence intensity of Figure 3a.

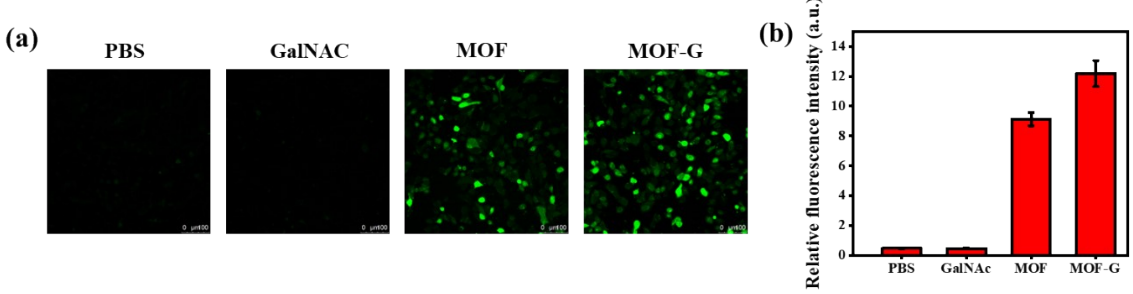


Figure S17. (a) CLSM images of Hepa1-6 cells treated under different treatments to evaluate ROS production based on DCFH-DA fluorescence intensity. (Scale bar = 100 μm) (b) The quantitative analysis of fluorescent intensity in Figure S14a.

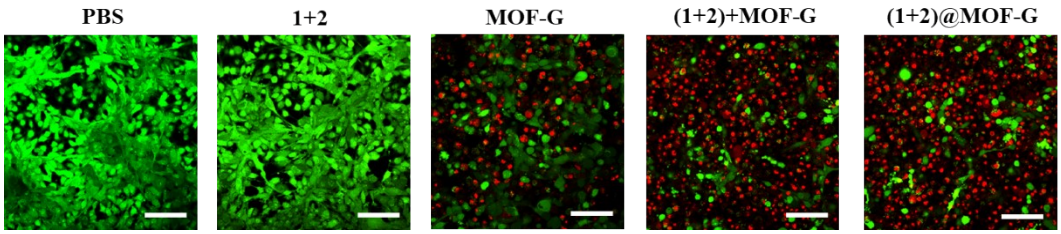


Figure S18. Live/dead cell staining assay of cells subjected to different treatments. Scale bars = 100 μm.

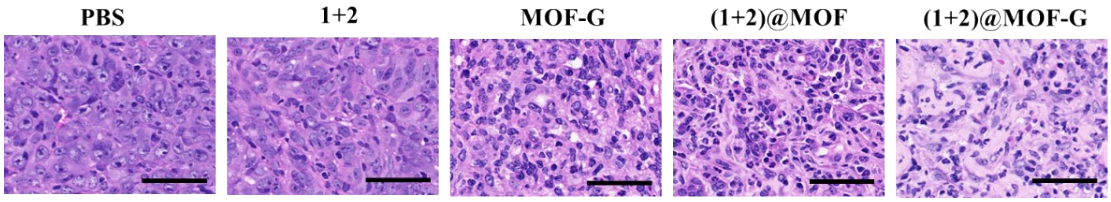


Figure S19. H&E staining of the four tumor slices with different treatments. Scale bars = 50 μm.

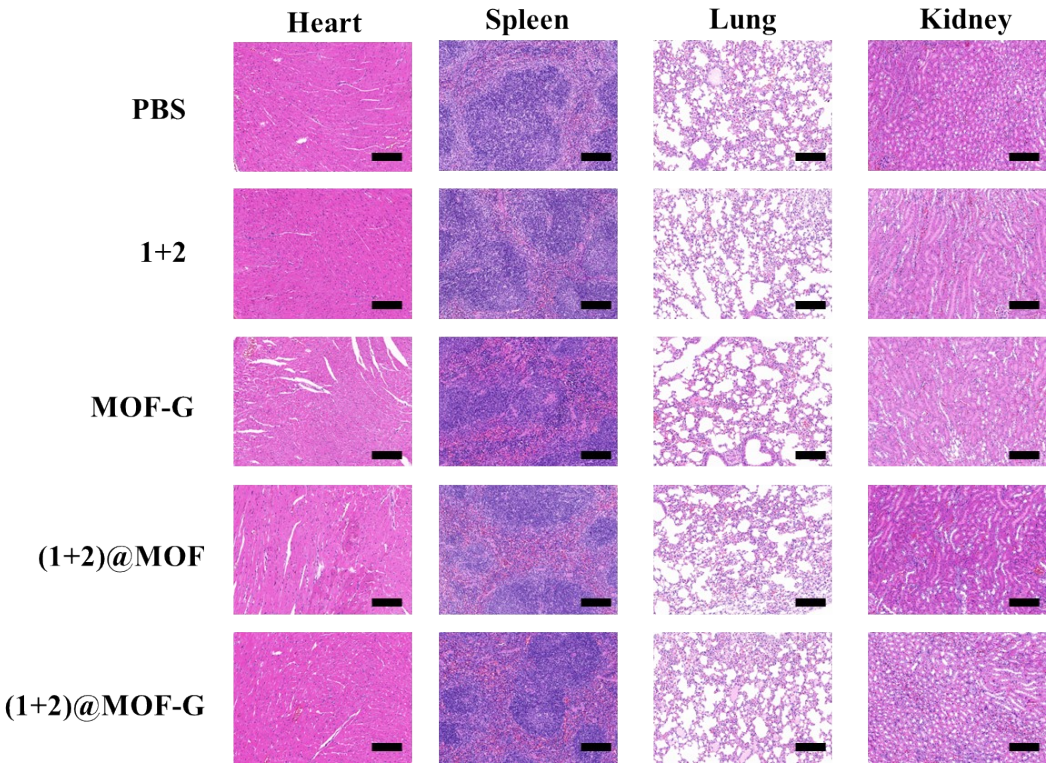


Figure S20. H&E staining of the four major organs with different treatments after 14 days. All scale bars are 150 μm.

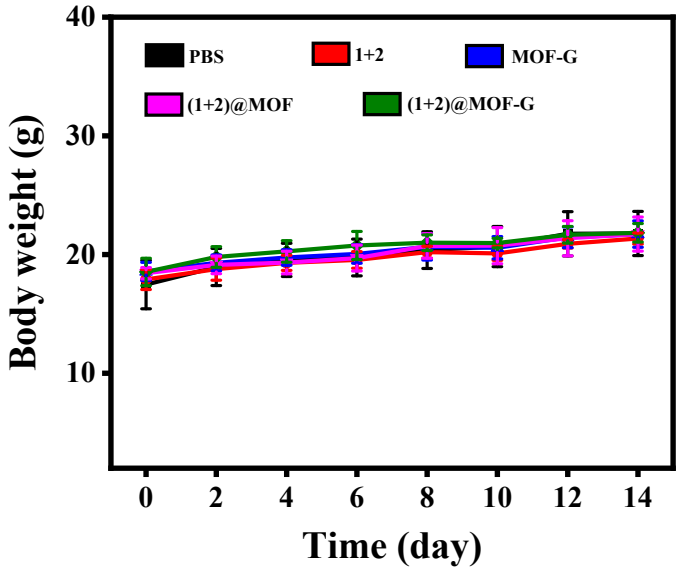


Figure S21. Body-weight changes of tumor-bearing mice within 14 days during treatment.

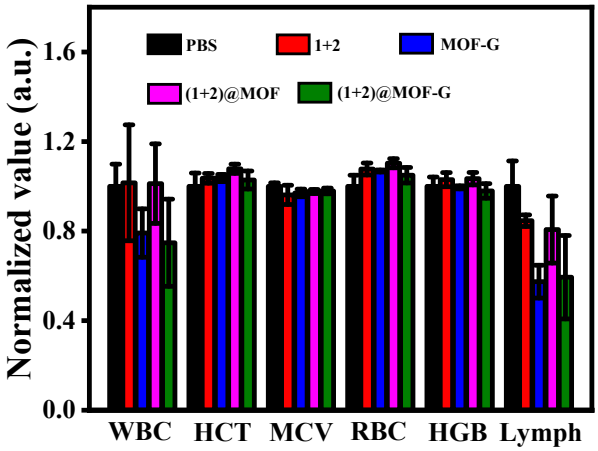


Figure S22. Hematological parameters after different treatments.

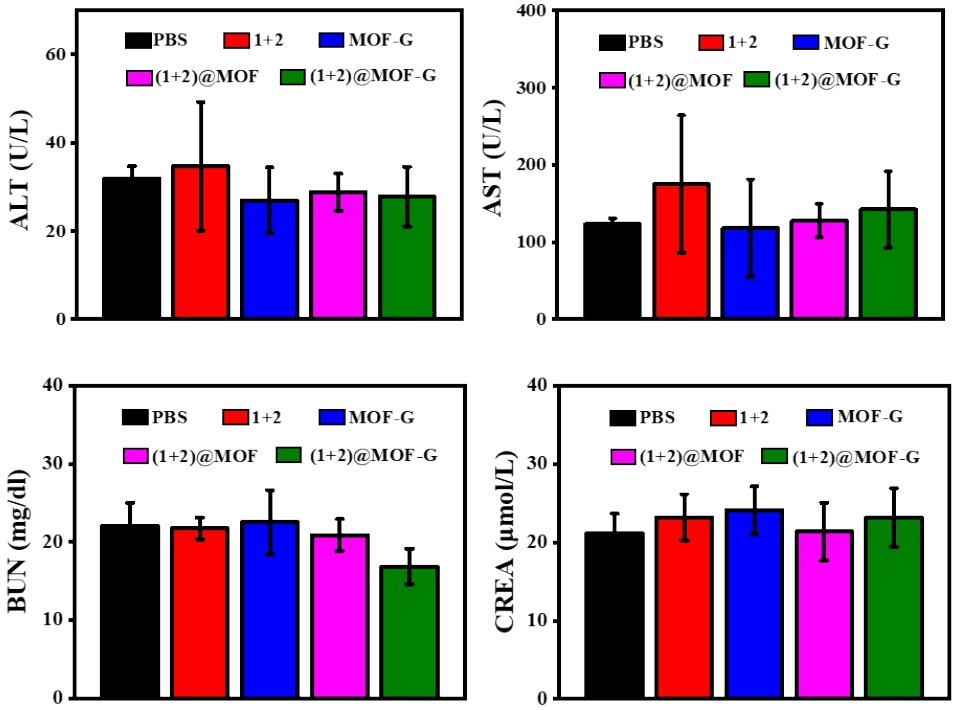


Figure S23. Blood biochemical parameters after different treatments.

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