Supporting Information

GSH-Triggered NO Releasing Nanoplatform based on a Covalent Organic Framework for "1 + 1 > 2" Synergistic Cancer Therapy

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EXPERIMENTAL SECTION

Materials and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Company. Calcein-AM/PI Double Stain Kit was purchased from Beyotime (Nantong, China). Reactive Oxygen Species Assay Kit (2',7,-dichlorofluorescin diacetate, DCFH-DA) and 3-Amino,4-aminomethyl-2′,7′-diflfluorescein diacetate (DAF-FM DA) were purchased from Beyotime (Nantong, China). Griess reagent was purchased from Beyotime (Nantong, China). 1,3-diphenylisobenzofuran (DPBF) and trypan blue were purchased from Tianjin Heowns Biochemical Technology Co., Ltd. Benzofuroxan (BFX) was purchased from Shanghai Titan Scientific Co., Ltd. Glutathione (GSH) was purchased from Energy Chemical (Shanghai, China).Hyaluronic acid (HA) was purchased from Shandong Freda Biotechnology Co., Ltd. All the other chemical reagents were of analytical grade and used without further purification. All starting materials and solvents, unless otherwise specified, were obtained from commercial resources and used without further purification. 1,3,5,7-Tetrakis(4-aminophenyl)adamantane (TAPA) and 5,10,15,20-tetrakis(4-benzaldehyde)porphyrin (TBAP) were synthesized using published procedures.Human breast cancer MCF-7 cells were obtained from Aoluo Biotechnology Co. Ltd. (Shanghai, China). Fetal bovine serum (FBS), DMEM, MEM, RPMI-1640 and PBS were purchased from Biological Industries (Beit Haemek, Israel).

Instruments

Fourier infrared spectrometer (Nicolet iS50 FT-IR) was used to characterize the infrared spectrum. Transmission electron microscopy (TEM, HT7700, Japan) was employed to characterize the morphologies of the nanoreactors. Powder X-ray diffraction (XRD) pattern was obtained on a Rigaku SmartLab SE X-Ray Powder Diffractometer with Cu Kα line focused radiation (λ = 1.5405 Å). Fluorescence spectra were obtained using a FLS-980 Edinburgh Fluorescence Spectrometer with a Xenon lamp. UV-vis spectroscopy was achieved with UV-1700 (Shimadzu, Japan).

The absorbance was measured in a microplate reader (Synergy 2, Biotek, USA) for the MTT assay. Confocal fluorescence imaging studies were performed using a TCS SP8 confocal laser scanning microscope (Leica, Germany). All pH measurements were performed with a digital pH-meter (pH-3e, LeiCi, China). In vivo fluorescence images were captured using live animal imaging system (IVIS Lumina III, US).

Synthesis of BFX@COF.

The nanoscale COF was firstly prepared using the method we reported.[34] Then 2 mg COF and 2 mg BFX were mixed in 4 mL aqueous solution in dark for 24 h. Then the above solution was centrifuged at 14000 rpm. The precipitate was washed twice with deionized water. The supernatant was collected to calculate the drug loading efficiency according to the standard curves.

Preparation of BFX@COF-HA

EDC (20 mg) and NHS (20 mg) were added to the aqueous solution of HA (40 mg) and kept in dark for 0.5 h. Then BFX@COF nanoparticles (2 mg) were added in the above-mentioned solution and stirred for 12 h. The obtained BFX@COF-HA were collected via centrifugation (14000 rpm, 10 min) and washed with water for subsequent use.

The stability of the BFX@COF-HA in the DMEM

The prepared BFX@COF-HA was dispersed in PBS, DMEM, MEM, and RPMI-1640 for 1, 2, 3, 4, 5, 6, and 7 days, then the particle size was measured.

Detection of extracellular NO

Classical Griess reagent was used to monitor the concentration of NO. According to the kit instructions, the OD values of $NaNO_2$ standard solution (1, 5, 10, 20 and 40 μM) was recorded using a microplate reader, and the standard curve of $NaNO_2$ was obtained.

(1) 1 mg BFX was added to 1 mL PBS solution of GSH (10 mM or 200 μM) and the pH value was adjusted to 5.4. Then concentrations of NO were measured at 0, 0.5, 1, 2, 3, 5 and 8 h according to the Kit instruction.

(2) BFX (0.1 mg) or BFX@COF-HA (1.2 mg) was added to 1 mL PBS solution of GSH (10 mM) and the pH value was adjusted to 5.4. After 24 hours, concentrations of

NO were measured according to the Kit instruction.

(3) BFX (0.1 mg) or BFX@COF-HA (1.2 mg) was added to 1 mL PBS solution of GSH (10 mM) and the pH value was adjusted to 5.4. Then concentrations of NO were measured at 0, 1, 5, 9 and 22 h according to the Kit instruction.

GSH consumption experiment.

9 mg BFX was added to 9 mL aqueous solution of GSH (2 mM). Then 500 μL of the above solution was taken at 0,2,4,8 and 12 h, respectively, and 70 μL DTNB (10mg/mL) was added to measure its UV absorption at 412 nm, repeated three times.

Detection of ROS.

DPBF (20μg/mL) was firstly mixed with COF-HA (0.025 mg/mL) or BFX@COF-HA (0.025mg/mL), respectively. After irradiated with 635 nm laser (0.625W/cm2) for different times (0.5 min, 1 min, 1.5 min, 2 min, 3 min), the absorption spectra of the solutions were determined via a UV-visible spectrophotometer.

Cell culture

MCF-7 cells were incubated in cell culture dishes with a diameter of 10 cm containing DMEM supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5 % CO_2.

Cellular uptake assay

（1）The preparation of IR808@COF-HA was as follows: 1 mg of COF-HA NPs and 1 μL of IR808 (10 mg/mL) were mixed in 1 mL of aqueous solution and stirred for 12 h. Then the obtained IR808@COF-HA was collected by centrifugation and washed three times for future use. MCF-7 cells and HL-7702 cells were inoculated into confocal dishes and incubated for 24 hours. Then the cells were added IR808@COF-HA (200μg/mL). After 5 hours, the cells were washed for two times as well as analyzed with CLSM (λ_{ex} = 780 nm, λ_{em} = 800 - 850 nm).

（2）MCF-7 cells and HL-7702 cells were dispersed in 96-well plate and incubated for 24 h. MCF-7 cells and HL-7702 cells were then incubated with 100μg/mL and 200μg/mL of the BFX@COF-HA, respectively. After 4 hours, fresh culture medium was replaced. Then 635 nm laser (0.625 W/cm^2) was employed on corresponding

groups for 10 min. Then the culture medium was removed from the 96-well, and 150 μL of MTT solution (0.5 mg/mL) was added. After incubated for 4 h, the MTT solution was removed, and 150 μL of DMSO was added. The absorbance at 490 nm was monitored by a microplate reader.

Flow cytometry analysis of NO generation

MCF-7 cells were cultured in cell dishes with DMEM for 24 h and randomly divided into 5 groups: PBS, BFX-1(30 μg/mL), BFX@COF-HA-1(200 μg/mL), BFX-2(30 μg/mL), BFX@COF-HA-2(200 μg/mL). Each group of cells incubated for 4h or 9h. Subsequently, each group of cells was collected through the centrifugal. After that, the cells were treated with DAF-FM DA for 20 min and washed with PBS for three times. Finally, MCF-7 cells were resuspended in 80 μL PBS for flow analysis.

Detection of intracellular ROS generation level

MCF-7 cells were cultured in confocal dishes overnight at 37 °C and divided into 5 groups: PBS, COF-HA, COF-HA+hν, BFX@COF-HA and BFX@COF-HA+hν. The different nanomaterials (200 μg/mL) in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) were added to the confocal dishes as well as incubated with 4 hours in anaerobic conditions, respectively. Then all MCF-7 cells further treated with fresh culture media containing DCFH-DA (1 μg/mL) for 20 min before irradiation. For irradiation groups, 635 nm laser (0.625 W/cm^2) was utilized to irradiate MCF-7 cells with 10 min. Subsequently, the cells were washed for three times as well as analyzed with CLSM (λ_{ex} = 488 nm, λ_{em} = 515 - 560 nm).

MTT assay

MCF-7 cells were dispersed in 96-well plate and incubated for 24 h, and divided into five groups: PBS, COF-HA, COF-HA+hν, BFX@COF-HA, BFX@COF-HA+hν, to incubate with 200 μL (200 μg/mL) corresponding agents for 4 h. The control group was PBS group. Then 635 nm laser (0.625 W/cm^2) was employed on corresponding groups for 10 min. After 7 h, fresh culture medium was added and further incubate for the night. Then the culture medium was removed from the 96-well, and 150 μL of MTT solution (0.5 mg/mL) was added. After incubated for 4 h, the MTT solution was

removed, and 150 μL of DMSO was added. The absorbance at 490 nm was monitored by a microplate reader.

Live/dead cell staining assay

MCF-7 cells were inoculated into confocal dishes and incubated for 24 hours. Then the cells were treated with PBS, COF-HA, COF-HA+hν, BFX@COF-HA, BFX@COF-HA+hν with a concentration of 200 μg/mL. After incubated with 4 h, the groups needed laser were irradiated at 635 nm laser (0.625 W/cm^2) for 10 min.

After incubation overnight, calcein-AM/PI probe was added to the confocal dishes and incubated for another 15 min. Finally, the cells were washed three times with PBS and imaged with CLSM (λ_{ex} =488 nm, λ_{em} = 500-560 nm).

Establishment of tumor model

Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU2021082). 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. Female nude mice (6 weeks old, ~16 g) were raised under normal circumstances of 12 h light and dark cycles and given access to food and water optionally. 1×10^7 MCF-7 cells in 100 μL of serum-free RPMI DMEM medium were injected subcutaneously into the right axillary region of the nude mice. After the tumor volume had reached approximately 75-100 mm^3, the mice were used into subsequent therapy experiments.

In vivo fluorescence imaging

The preparation of IR808@COF-HA was as follows: 1 mg of COF-HA NPs and 1 μL of IR808 (10 mg/mL) were mixed in 1 mL of aqueous solution and stirred for 12 h. Then the obtained IR808@COF-HA was collected by centrifugation and washed three times for future use. IR808@COF-HA (50 μL, 4 mg/mL) were injected into nude mice through the caudal vein. At different time points of 0, 4, 12, 24, 48, 72h, after injection, the fluorescence intensity of the mice tumor was recorded through a live body imaging system.

In vivo antitumor experiment

MCF-7 breast cancer bearing mice were divided into five groups: PBS, COF-HA, COF-HA+hν, BFX@COF-HA, BFX@COF-HA+hν. Nanoparticles (10 mg/kg) were injected by tail vein. After 12 hours, for irradiation groups, 635 nm lasers（0.625 W/cm^2 ）were utilized to irradiate 10 min. After that, the tumor growth and body weight change situations of the mice were recorded within 14 days.

Detection of ROS level in tumors during the therapy

MCF-7 breast cancer bearing mice were divided into three groups: PBS, COF-HA (500μg) and BFX@COF-HA (500μg). After mixing the materials with DCFH-DA probe (2μL 10mM), they were injected into the tumor and were irradiated under 635 nm lasers（0.625 W/cm^2 ） for 10 min. Then fluorescence intensity of the mice tumor was recorded through a live body imaging system. (λ_{ex} = 488 nm, λ_{em} =525 nm).

Detection of NO level in tumors during the therapy

MCF-7 breast cancer bearing mice were divided into three groups: PBS, BFX (75μg) and BFX@COF-HA (500μg). After mixing the materials with DAF-FM probe (3μL, 5mM), they were injected into the tumor. After 3 hours, fluorescence intensity of the mice tumor was recorded through a live body imaging system. (λ_{ex} =495 nm, λ_{em} = 515 nm).

Histopathological analysis

MCF-7 breast cancer bearing mice were divided into five groups: PBS, COF-HA, COF-HA+hν, BFX@COF-HA and BFX@COF-HA+hν. Nanoparticles (10 mg/kg) were injected by tail vein. After 12 hours, COF-HA+hν and BFX@COF-HA+hν were irradiated under 635 nm lasers （0.625 W/cm^2 ）for 10 min. After treatment, the representative tumor-bearing mice from different treatment groups were sacrificed and the tumors and five major organs (liver, lung, spleen, kidney, and heart) were harvested to use for hematoxylin and eosin (H&E) staining.

Blood biochemical parameters and hematological parameters analysis

MCF-7 breast cancer bearing mice were divided into five groups: PBS, COF-HA, COF-HA+hν, BFX@COF-HA and BFX@COF-HA+ hν. Nanoparticles (10 mg/kg)

were injected by tail vein. After 12 hours, COF-HA+hν and BFX@COF-HA+hν were irradiated under 635 nm lasers（0.625 W/cm^2 ） for 10 min. After treatment, Blood routine and biochemical analysis were performed after the blood was collected from the retroorbital plexus.

SUPPORTING FIGURES

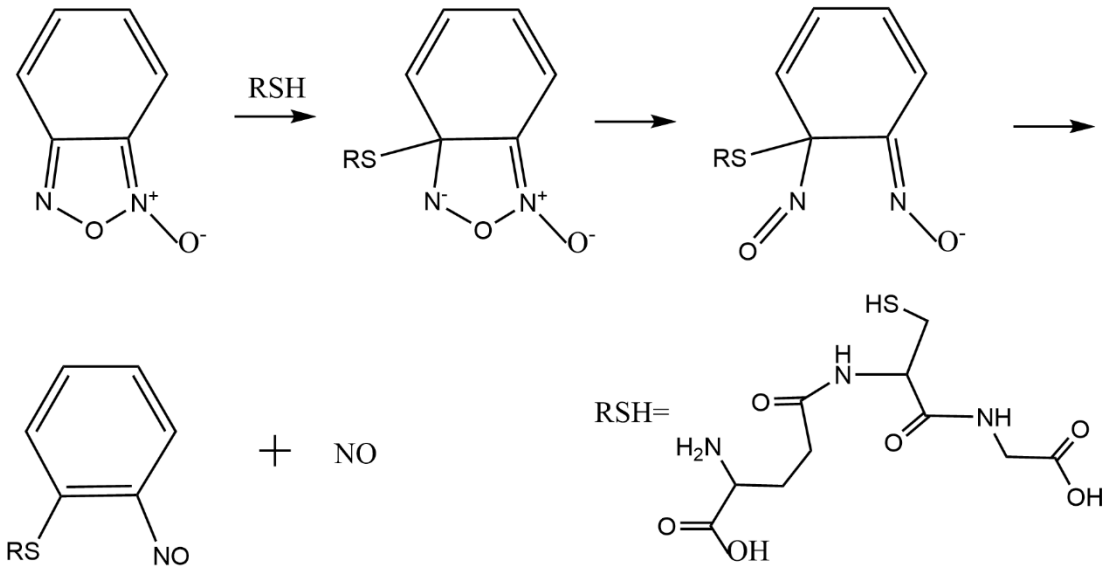

Figure S1. Mechanism of GSH triggered NO release from BFX**.**

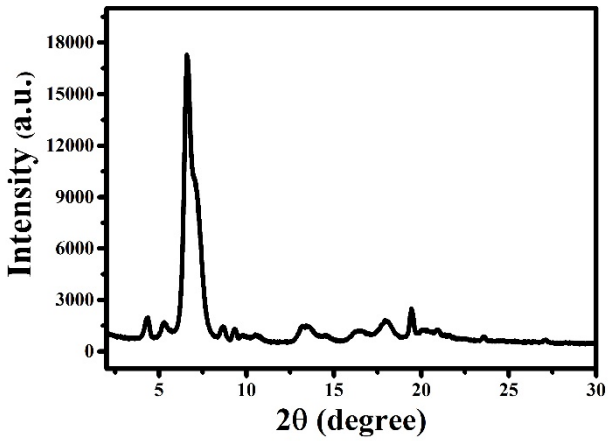

Figure S2. PXRD pattern of COF.

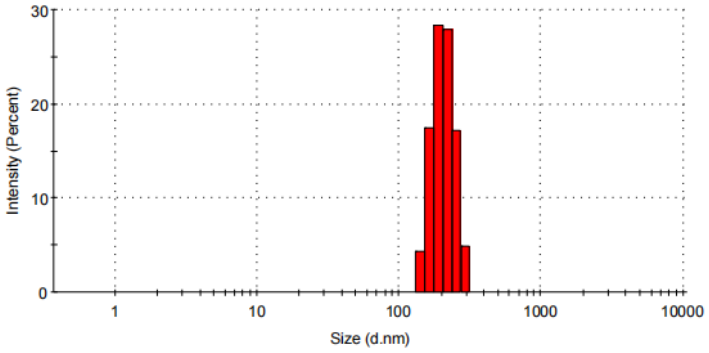

Figure S3. DLS size distribution of COF.

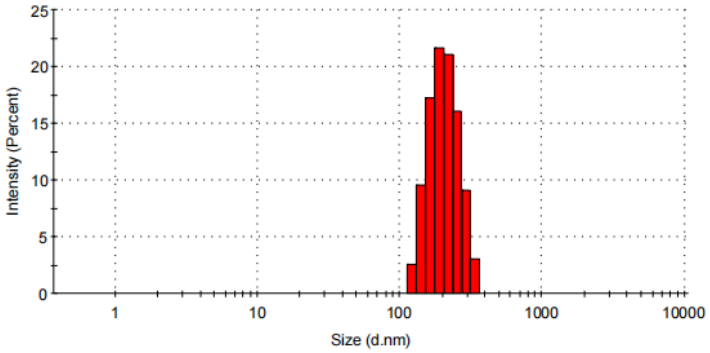

Figure S4. DLS size distribution of BFX@COF.

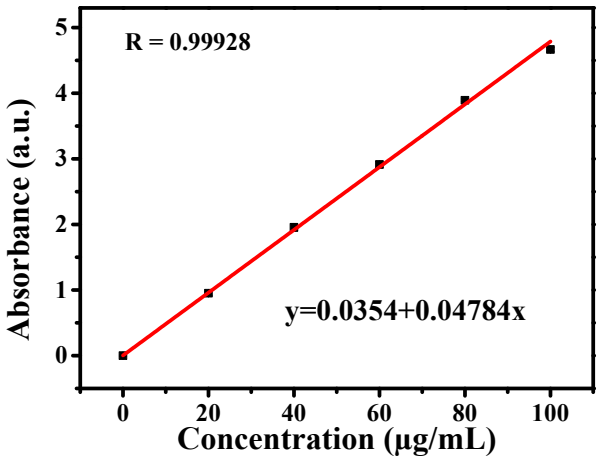

Figure S5. Linear relationships between the absorbance intensity of BFX and the BFX concentration. The error bar is the standard deviation from the mean (n = 3).

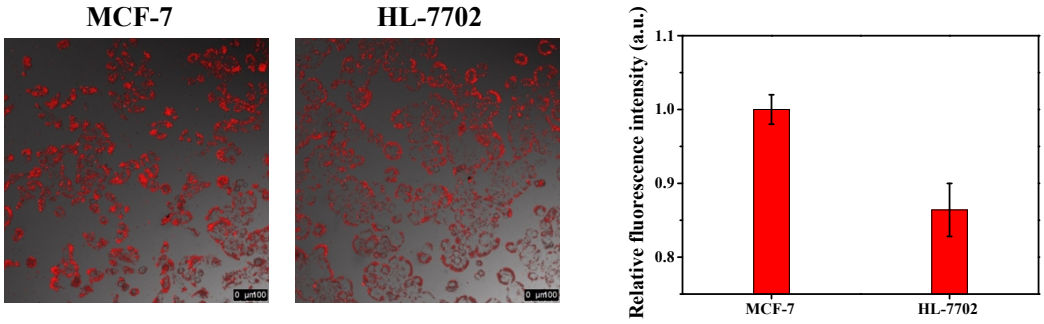

Figure S6. Cellur uptake evaluation of BFX@COF-HA to cance cell and normal cell.

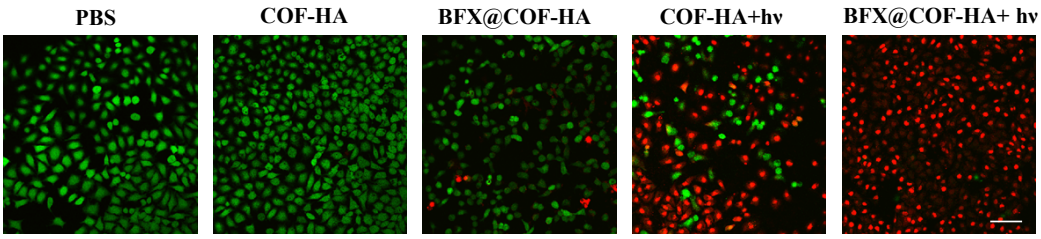

Figure S7. Live/dead cell staining assay of cells subjected to different treatments**.** Scale bars = 100 μm.

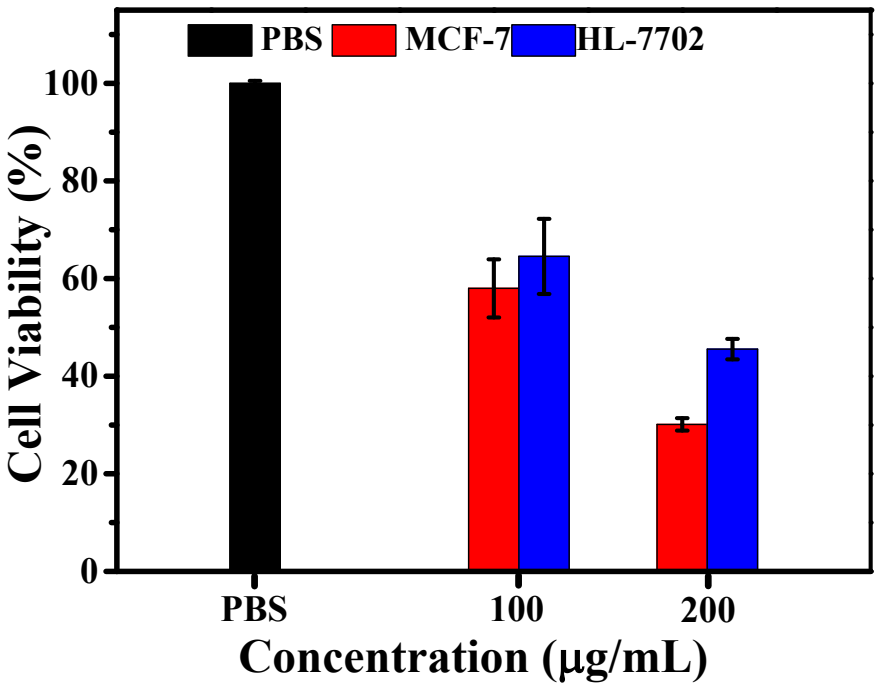

Figure S8. The MTT assay of cancer cell and normal cell treated with different concentration of BFX@COF-HA+hν.

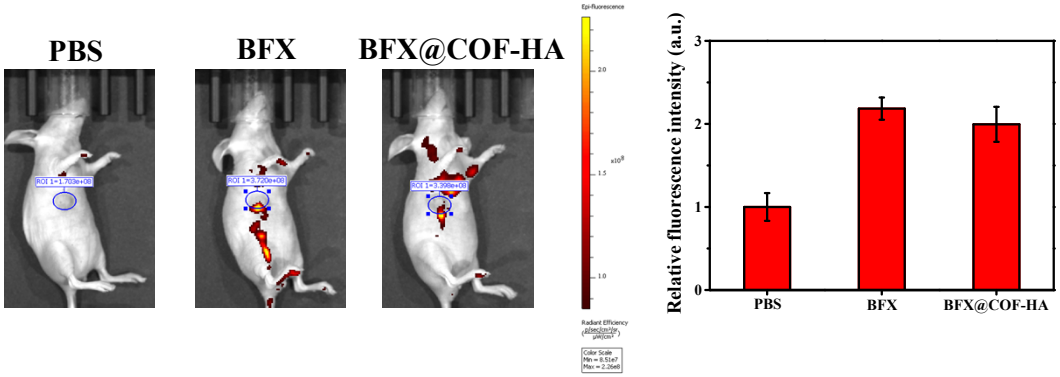

Figure S9. *In vivo* imaging based on the fluorescence of DAF-FM to evaluated NO level.

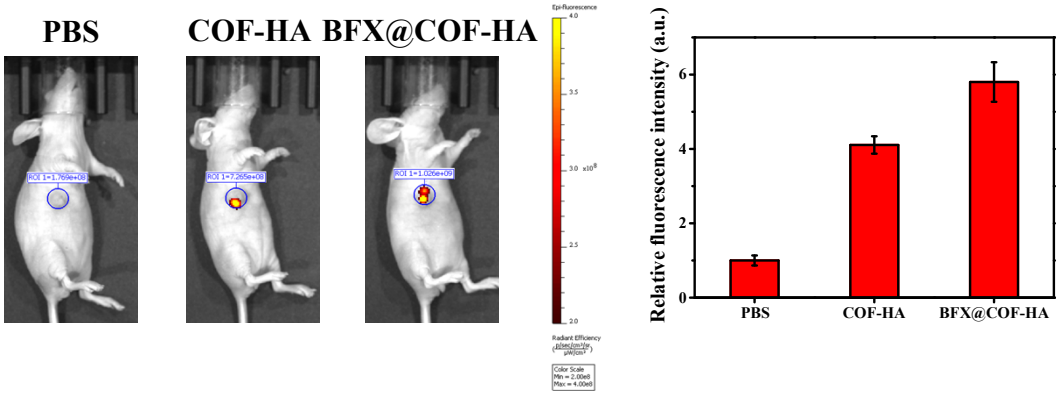

Figure S10. *In vivo* imaging based on the fluorescence of DCFH-DA to evaluated ROS level.

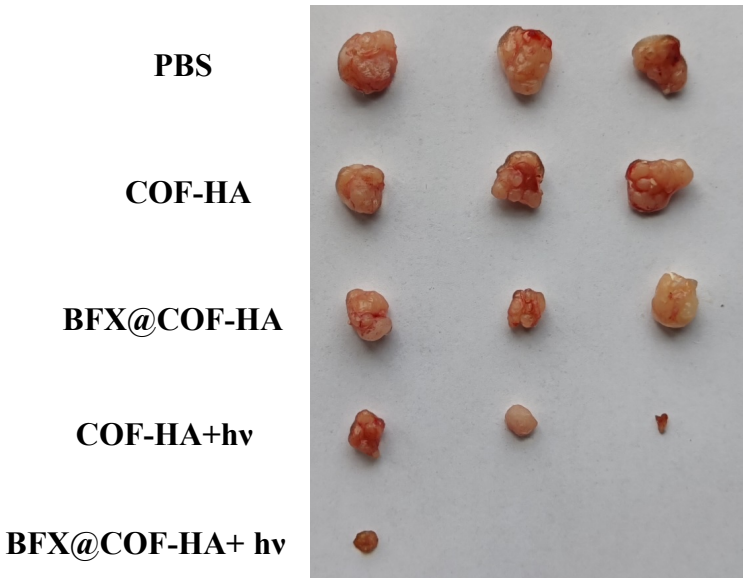

Figure S11. Picture of tumors dissected on the 14th day after different treatments.

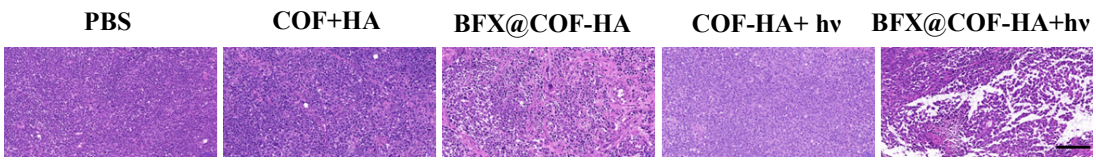

Figure S12. H&E staining images of tumor slices. All scale bars are 150 μm.

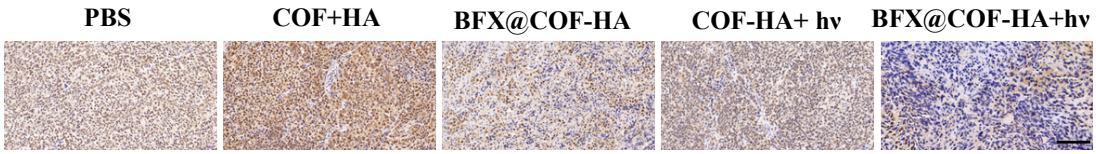

Figure S13. Antigen Ki-67 immunofluorescence staining images of tumor slices. All scale bars are 150 μm.

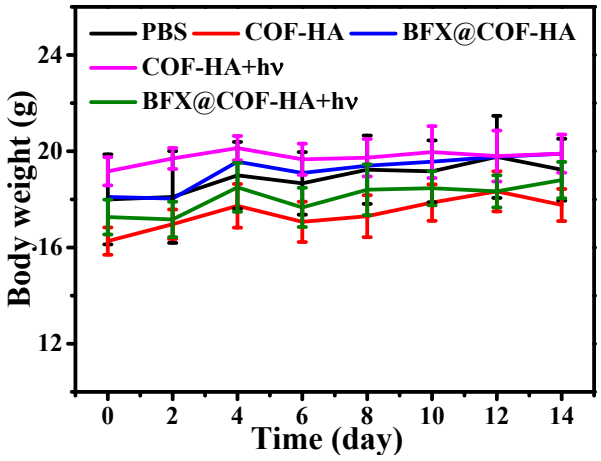

Figure S14. Body-weight changes of mice within 14 days during treatment. The error bar is the standard deviation from the mean (n = 3)

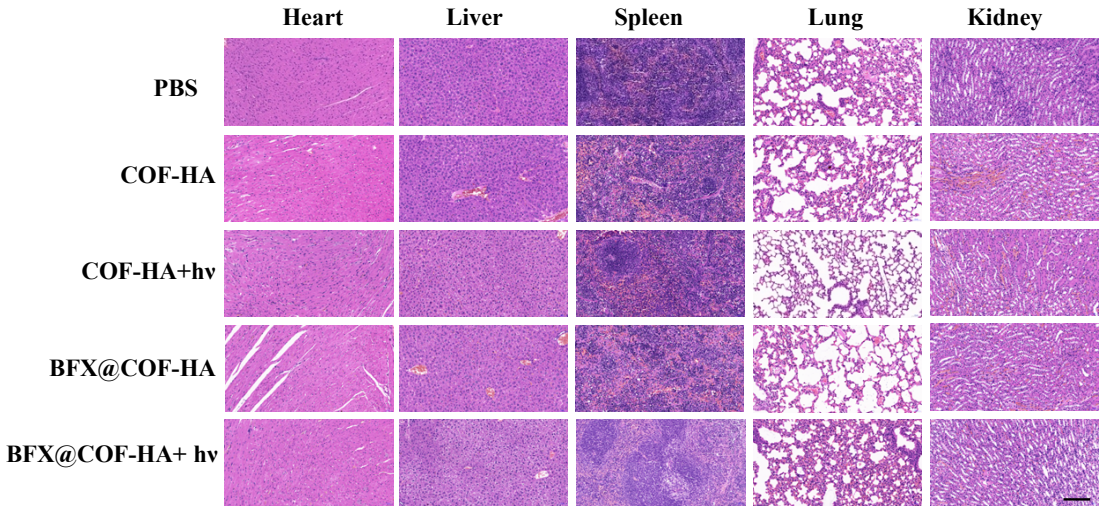

Figure S15. H&E staining of the five major organs with different treatments after 14 days. All scale bars are 150 μm.

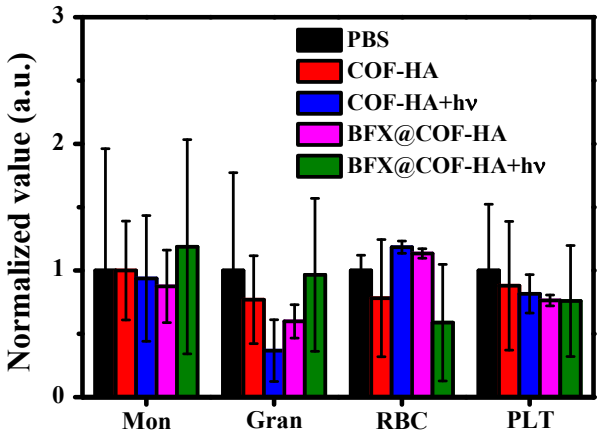

Figure S16. Hematological parameters after different treatments for 7 days. The blood was collected for detection of the levels of Mon, Gran, RBC, and PLT. The error bar is the standard deviation from the mean (n = 3).

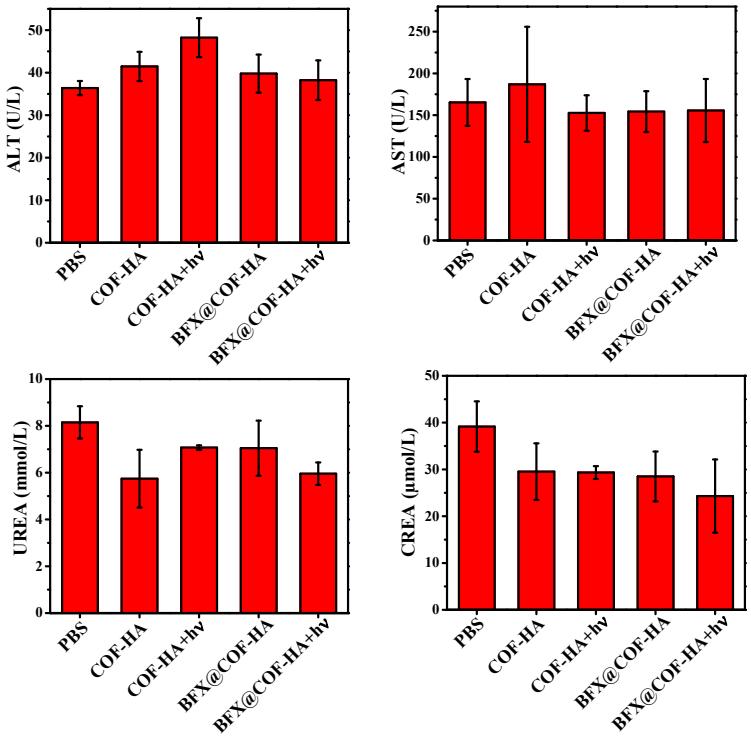

Figure S17. Blood biochemical parameters after different treatments for 7 days. The blood was collected for detection of the levels of ALT, AST, UREA and CREA. The error bar is the standard deviation from the mean (n = 3).