Supporting Information

Nanodrug Combining Chemotherapy and Ferroptosis Mediated cGAS-STING Activation for Potent Antitumor Immunity

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1. Experimental sections

1.1 Materials, cell lines and animals

Tannic acid (TA) and propidium iodide (PI) were obtained from Energy Chemical (Shanghai, China). Doxorubicin Hydrochloride (DOX·HCl) was purchased from Prosenreite Biomedical Technology Co., Ltd. (Tianjin, China). 3, 3', 5, 5'-Tetramethylbenzidine (TMB), 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich (China). Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was sourced from Alab Chemical Technology (Shanghai, China). The ROS Green™ H_2O_2 Probe was purchased from Maokang Co., Ltd. (Shanghai, China). The BCA protein assay kit and enhanced ATP Assay Kit were supplied by Beyotime Biotechnology (Shanghai, China). HMGB1 Rabbit mAb and Calreticulin Rabbit pAb antibodies were obtained from ABclonal (Wuhan, China). DMEM was sourced from Gibco (USA), and fetal bovine serum was provided by GEMINI (USA). Penicillin-streptomycin solution (100×) was obtained from Biosharp (Beijing, China).

The 4T1 and MDA-MB-231 cell lines were provided by the Cell Bank, Chinese Academy of Sciences.

BALB/c and C57BL/6J mice were supplied by Sibeifu Biological Technology Co., Ltd. (Beijing, China).

1.2 Instruments

Nanodrug images were viewed by a Hitachi H-7650 transmission electron microscope (TEM). The UV-Vis spectra were determined by Thermo (BioMate 3S) UV-Vis spectrophotometer. The fluorescence spectra were characterized with F97pro fluorescence spectrophotometer and multifunctional enzyme labeling instrument (SpectraMax iD3). The fluorescence images in cells were completed by an Olympus FV1200 confocal laser scanning microscope (CLSM). Animal images were obtained with a PerkinElmer IVIS Lumina III animal imaging apparatus.

1.3 Preparation of the FTNPs

FTNPs were prepared using a one-pot method. $FeCl_3 \cdot 6H_2O$ and TA were dissolved in 1 mL of deionized water (ddH_2O) at room temperature to a concentration of 4.8 mM. 200 μL of the prepared $FeCl_3 \cdot 6H_2O$ and TA solution was added to 2 mL of ddH_2O (pH 7.0), and the mixture was stirred for 1 h. The resulting precipitate was washed three times with ddH_2O.

1.4 Characterization

The morphologies of FTNPs and DFTNPs were observed using TEM. The UV-Vis spectra were obtained from a UV-Vis spectrophotometer. The fluorescence spectra were determined with a fluorescence spectrophotometer. Dynamic laser scattering (DLS) was used to obtain the zeta potential of the nanodrugs.

1.5 Detection of DOX release

The degradation of the DFTNPs was monitored using the multifunctional enzyme labeling instrument. DFTNPs were added to PBS (pH 5.5, 7.0 and 10 mM GSH) and then incubated for 150 min. The multifunctional enzyme labeling instrument was also used to measure the changes in the absorption spectra before and after particle degradation.

1.6 Cell culture

The cells used in this experiment were MDA-MB-231 cells (Human breast cancer cells) and 4T1 cells (Mouse breast cancer cells). These cells were seeded in T25 flask at 37 °C in a 5% CO_2 incubator, using DMEM supplemented with 10% FBS and 1% penicillin-streptomycin.

1.7 Cell uptake observation by CLSM

The uptake of DFTNPs by cells was evaluated by CLSM. Briefly, MDA-MB-231 or 4T1 cells were seeded into confocal dishes (1×10^6/well). The cells were incubated with DFTNPs for 1 h, 2 h and 4 h, followed by two washes with PBS. They were then fixed with 4% paraformaldehyde and stained with Hoechst 33342.

1.8 MTT

MDA-MB-231 or 4T1 cells were seeded into 96-well plates and cultured for 24 h. FTNPs, free DOX or DFTNPs were diluted in DMEM medium and incubated with the cells for 24 h or 48 h. Subsequently, 100 μL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h. Afterward, the medium was carefully removed, and 100 μL of DMSO

was added to each well to dissolve the formazan crystals. The optical density (OD) was measured at 570 nm using a microplate reader, and cell viability was calculated accordingly.

1.9 Live/dead cell staining

MDA-MB-231 cells were inoculated into confocal dishes and incubated for 24 h. Then the cells were treated with PBS, FTNPs, DOX or DFTNPs for 24 h. After treatment, diluted Calcein AM and PI staining solution were added to the confocal dishes in the dark and incubated at 37 °C for 30 min. Then, cellular fluorescence was detected by CLSM.

1.10 Apoptosis assay

MDA-MB-231 cells were seeded in 6-well plates and incubated for 24 h. The cells were treated with PBS, FTNPs (100 μg/mL, 1 mL), free DOX or DFTNPs (DOX: 2 μg/mL, 1 mL) for 24 h and the cell status was assessed. Then, the cells were collected and resuspended in PBS. The cells were stained with Annexin V-FITC for 15 min and analyzed using flow cytometry.

1.11 Wound healing assay

MDA-MB-231 cells were inoculated in confocal dishes and incubated for 24 h. Then a scratch was created using a pipette tip, and images were captured under a microscope at 40× magnification. The cells were treated with PBS, FTNPs, free DOX or DFTNPs for 24 h. Subsequently, images were taken again under the microscope at 40× magnification to assess cell

migration.

1.12 Lipid peroxidation (LPO) detection

MDA-MB-231 cells were seeded into confocal dishes and incubated for 24 h. The cells were then treated with medium containing PBS, FTNPs (100 μg/mL, 1 mL), free DOX or DFTNPs (DOX: 2 μg/mL, 1 mL) for an additional 24 h. Finally, the cells were stained with the Liperfluo fluorescence probe for 30 min and washed with PBS. Green fluorescence was subsequently captured using CLSM.

1.13 Mitochondrial membrane potential (MMP) assay

First, MDA-MB-231 cells were attached to the confocal dish. Fresh medium containing drugs (FTNPs, DOX or DFTNPs) was added to the respective wells. After 24 h of incubation, Rhodamine 123 was added, and the cells were cultured for an additional 30 min. After being washed with PBS, the cells were imaged by CLSM.

1.14 Immunogenic cell death (ICD) induced by the DFTNPs

To evaluate ICD induced by chemotherapy and ferroptosis in tumor cells, we assessed the surface expression of calreticulin (CRT), the extracellular release of high mobility group box 1 (HMGB1), and the secretion of adenosine triphosphate (ATP) in vitro.[1,2] For immunofluorescence detection of CRT expression, MDA-MB-231 cells were seeded in confocal dishes and incubated overnight. The cells were then treated with medium containing the drugs and incubated for 24 h.

Following treatment, the cells were washed twice with TBS, fixed with 4% paraformaldehyde, and incubated with anti-CRT antibody for 30 min. Afterward, the cells were incubated with a Cy3-conjugated secondary antibody for an additional 30 min. Finally, the cells were stained with Hoechst 33342 for 5 min and observed using CLSM. Similarly, HMGB1 was detected using similar methods.

The effect of the drugs on ATP secretion of MDA-MB-231 cells was tested using an ATP Assay Kit. MDA-MB-231 cells were seeded into T25 flasks, and the supernatant was collected after treatment. The extracellular ATP content was measured according to the manufacturer's protocol.

1.15 Western blot

Western blot was used to detect the expression of ICD related proteins. MDA-MB-231 cells were seeded into 6-well plates and incubated for 24 h with media containing FTNPs, free DOX or DFTNPs, respectively. Next, the cells were harvested and lysed in RIPA lysis buffer. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) based on molecular weight and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% skim milk and incubated overnight at 4 °C with primary antibodies (CRT, HMGB1 and GAPDH). Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1.5 h at room temperature. The bands

were observed by ECL chemiluminescence.

1.16 Detection of IFN-γ, IFN-β, TNF-α and IL-6

BALB/c mice models were established and randomly divided into four groups: PBS, FTNPs (6.5 mg/kg, 100 μL), DOX and DFTNPs (DOX: 10 mg/kg, 100 μL). The drugs were administered every 3 days, and serum was collected from the mice on day 8. The levels of IFN-γ, IFN-β, TNF-α and IL-6 were quantified using ELISA kits according to the manufacturer's instructions.

1.17 Detection of mature dendritic cells (DC) in vitro

Bone marrow cells were extracted from 6-week-old C57BL/6J mice and resuspended in DMEM supplemented with 10% FBS. The cells were plated in 12-well plates and cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/mL, 1 mL) and IL-4 (20 ng/mL, 1 mL). The medium was replenished with cytokines on days 3 and 5 of culture. MDA-MB-231 cells were seeded in 6-well plates and incubated for 24 h. The old medium was replaced with medium containing drugs (DOX, FTNPs or DFTNPs) and incubated for an additional 24 h. The collected medium was used to DC for 48 h. DC cultured in 12-well plates were transferred to petri dishes, washed three times with TBS, and fixed with 4% paraformaldehyde for 15 min. The cells were blocked with 2% BSA at 37 °C for 30 min, followed by the addition of an anti-CD80 antibody and overnight incubation at 4 °C. After rewarming for 15 min, the

samples were washed and incubated with a Cy3-conjugated secondary antibody at 37 °C for 1 h in the dark. The cells were stained with Hoechst 33342 for 5 min, and fluorescence was detected using CLSM. CD86 was detected using the same protocol as CD80.

1.18 Hemolysis assay

Fresh blood (20 μL) from BALB/c mice was used for hemolysis studies to assess biosafety. Briefly, the blood was washed with PBS and centrifuged for red blood cell collection (3000 rpm, 15 min). Then, FTNPs, free DOX or DFTNPs with different concentrations (125 μg/mL, 250 μg/mL, 500 μg/mL, 750 μg/mL and 1000 μg/mL) were incubated with red blood cell suspensions for 4 h. The hemolysis rate was calculated based on the absorbance at 545 nm.

1.19 In vivo distribution analysis of DFTNPs in BALB /c mice

DFTNPs-Ce6 (Ce6: 2 mg/kg, 100 μL) was injected via the tail vein of BALB/c mice after the establishment of tumor models. Fluorescence changes at the tumor site were monitored over time to assess the biodistribution of the DFTNPs.

2. Supplementary figures

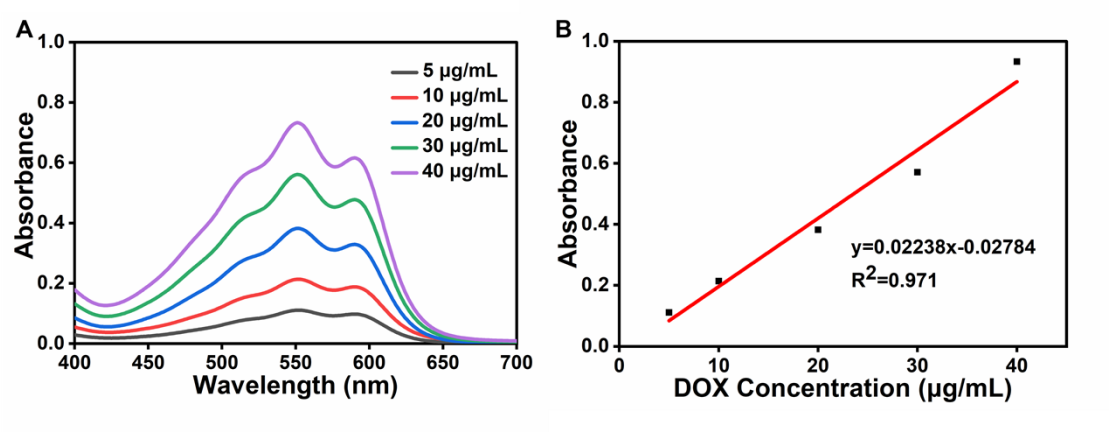

Fig. S1. (A) UV-Vis spectra of DOX with different concentrations. (B) Standard calibration curve of DOX at 551 nm.

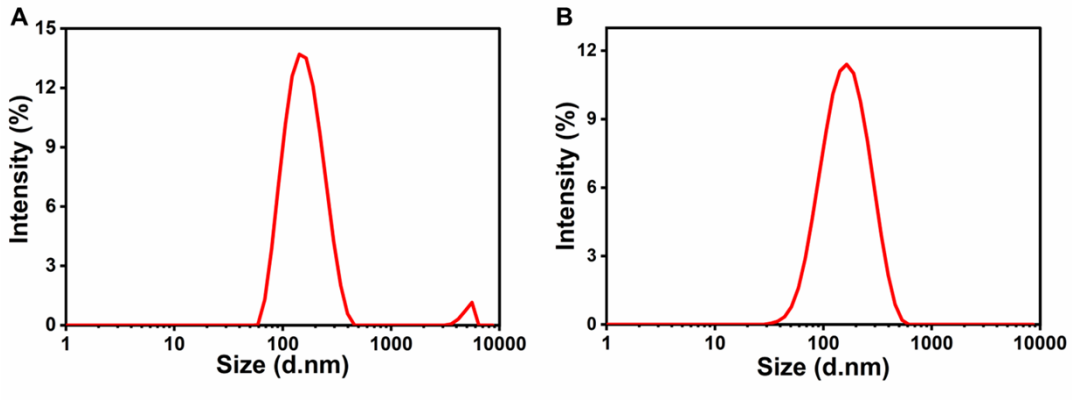

Fig. S2. The particle size distribution of DFTNPs (A) and FTNPs (B).

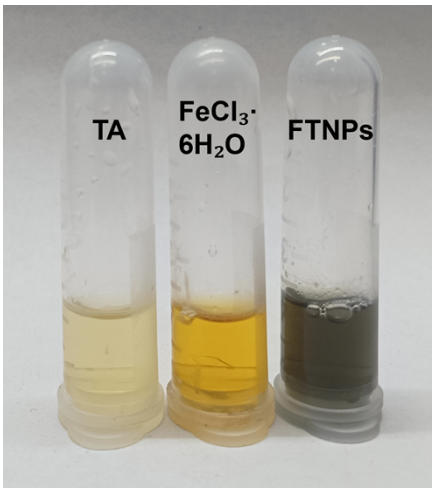

Fig. S3. The color of TA, $FeCl_3 \cdot 6H_2O$ and FTNPs in PBS (pH 7.4).

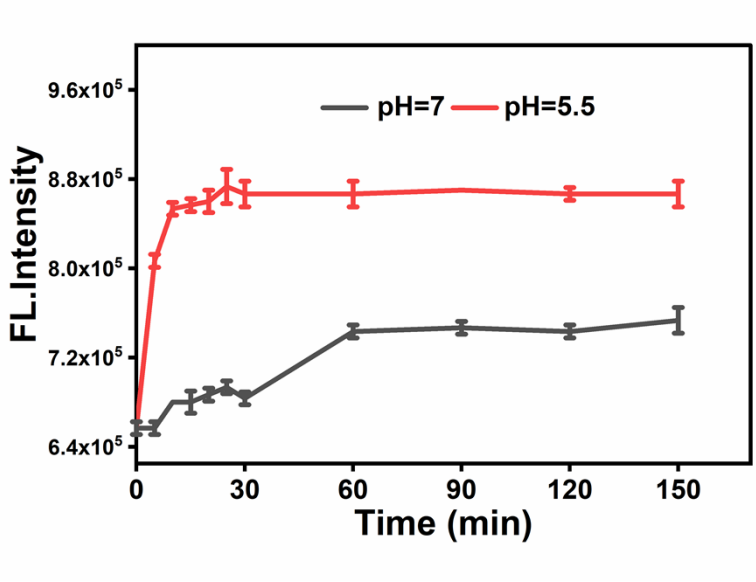

Fig. S4. Fluorescence intensity of DOX over different time intervals at pH 7 and pH 5.5. λ_{ex} = 488 nm.

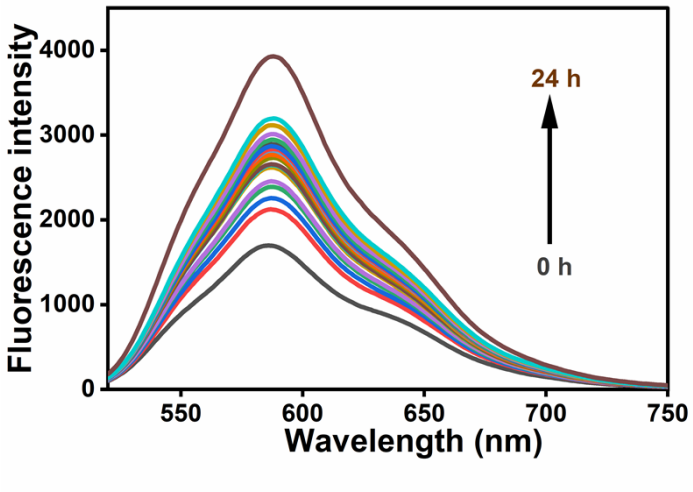

Fig. S5. Fluorescence intensity of DOX at pH 5.5 for 24 h. λ_{ex} = 488 nm.

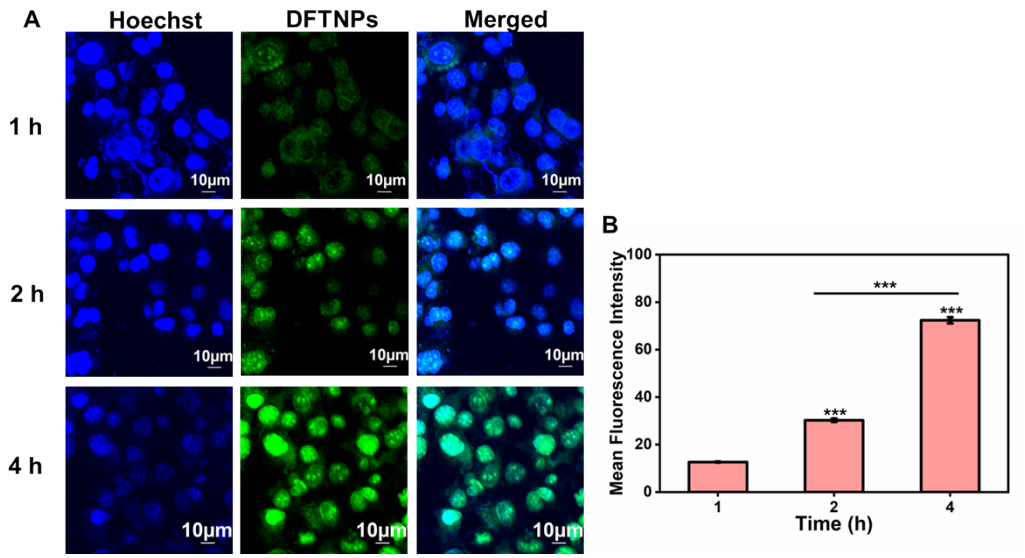

Fig. S6. (A) CLSM images of 4T1 cells incubation with DFTNPs for 1 h, 2 h and 4 h. Scale bar, 10 μm. (B) Quantitative analysis of the mean fluorescence intensity of DFTNPs from the different treatment groups in (A). Bases for error bars were SD ($n = 3$), ***$p < 0.001$.

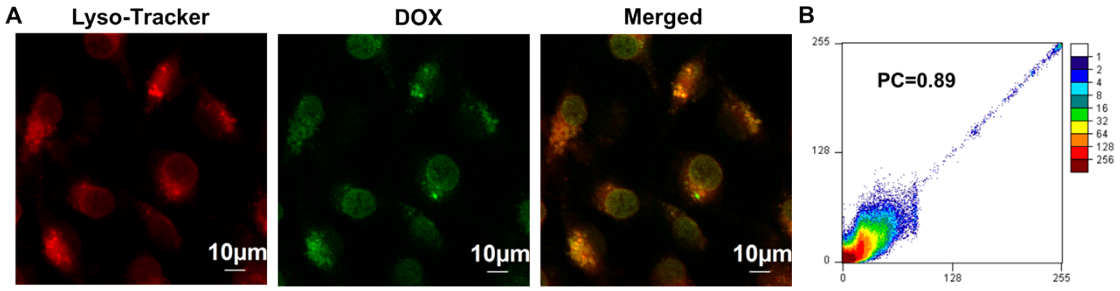

Fig. S7. (A) Fluorescence images of DFTNPs co-localized with Lyso-Tracker Green in MDA-MB-231 cells after 4 h. Scale bar, 10 μm. (B) Person's colocalization coefficient (PC).

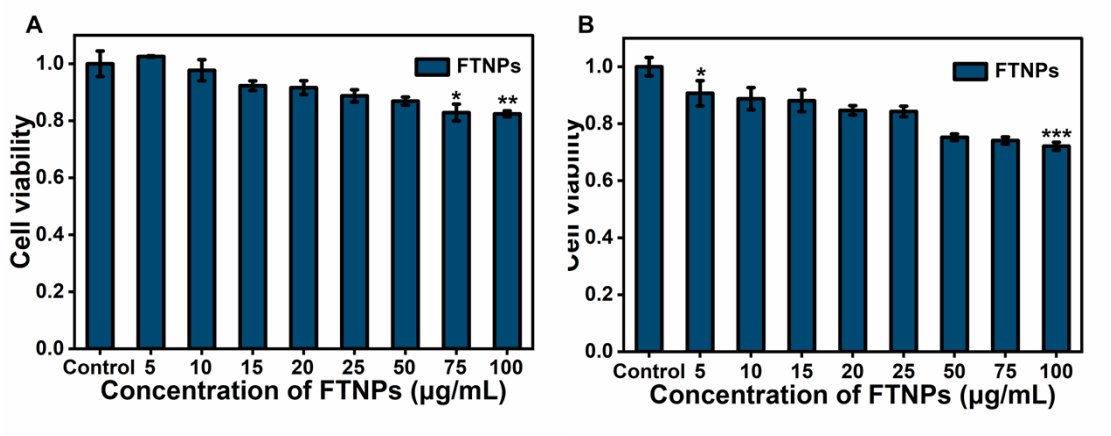

Fig. S8. (A) Cell viability of MDA-MB-231 cells treated with different concentrations of FTNPs for 24 h. (B) Cell viability of MDA-MB-231 cells treated with different concentrations of FTNPs for 48 h. Bases for error bars were SD (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001.

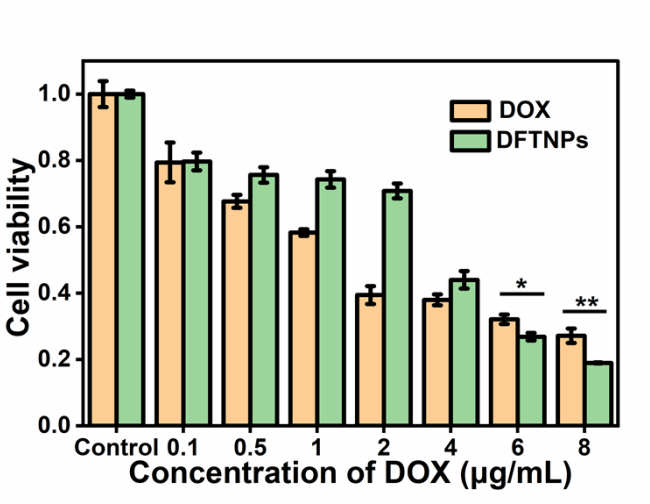

Fig. S9. Cell viability of MDA-MB-231 cells treated with different concentrations of DOX and DFTNPs for 48 h. Bases for error bars were SD (n = 3), *p < 0.05, **p < 0.01.

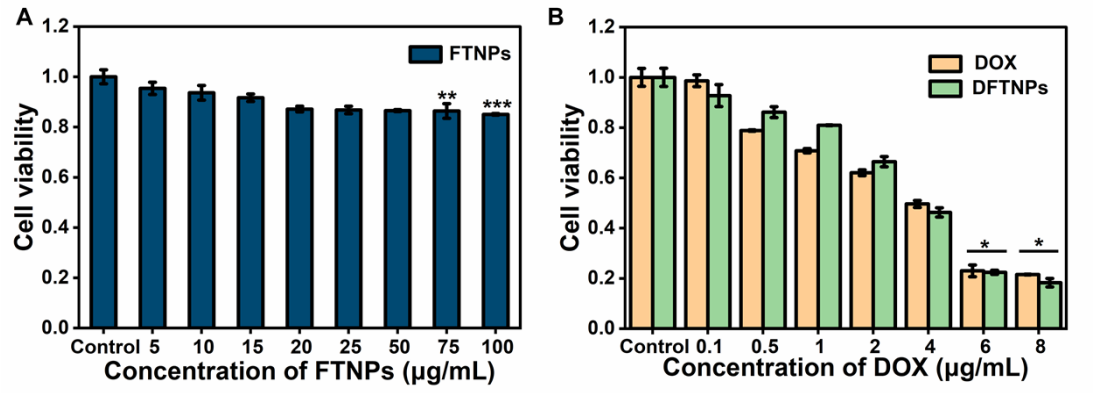

Fig. S10. (A) Cell viability of 4T1 cells treated with different concentrations of FTNPs for 24 h. (B) Cell viability of 4T1 cells treated with different concentrations of DOX and DFTNPs for 24 h. Bases for error bars were SD (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001.

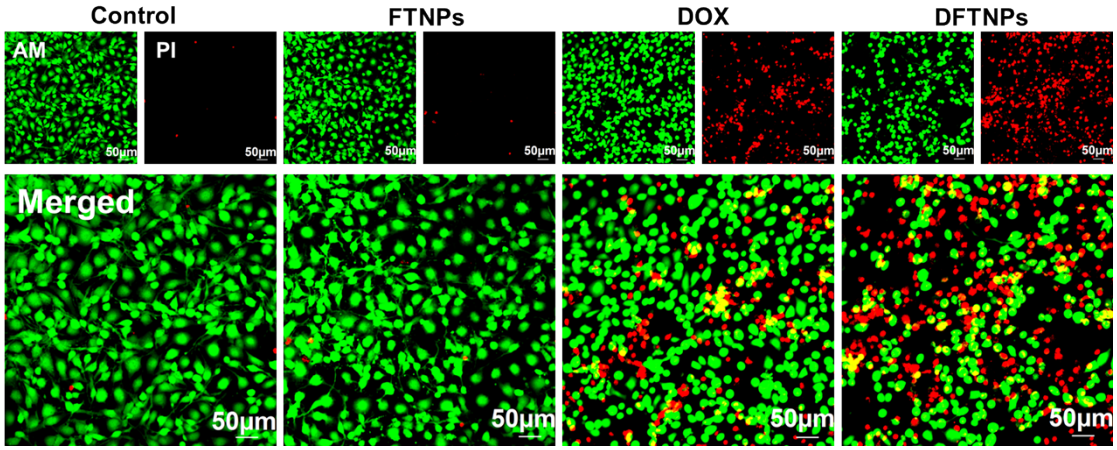

Fig. S11. Live/dead cell staining of MDA-MB-231 cells after incubation with different preparations for 24 h. Live cells: green fluorescence of AM; Dead cells: red fluorescence of PI. Scale bar, 50 μm.

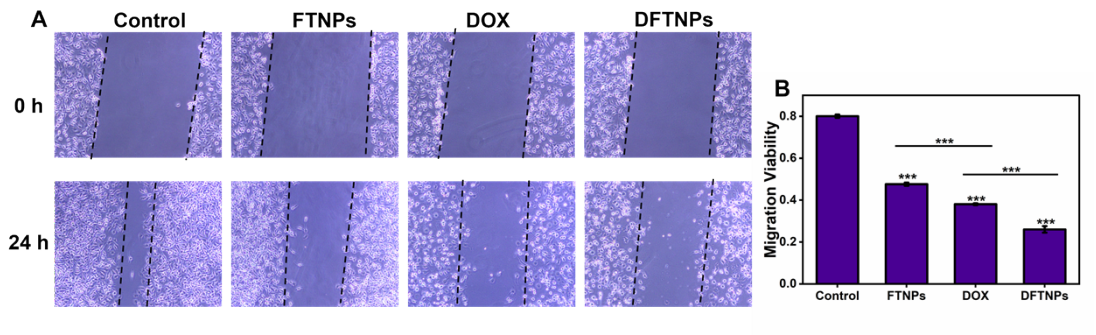

Fig. S12. (A) The wound healing photos of MDA-MB-231 cells treated with PBS, FTNPs, DOX and DFTNPs at 0 h and 24h. (B) Migration viability of MDA-MB-231 cells in different treatment groups. Bases for error bars were SD (n = 3), ***p < 0.001.

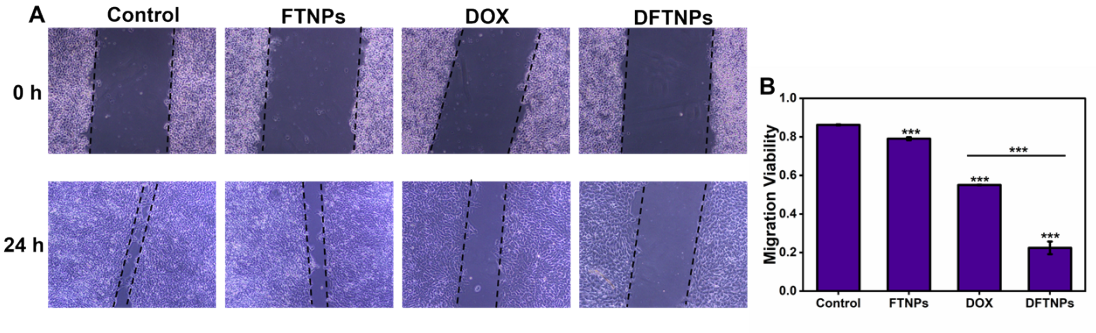

Fig. S13. (A) The wound healing photos of 4T1 cells treated with PBS, FTNPs, DOX and DFTNPs at 0 h and 24h. (B) Migration viability of 4T1 cells in different treatment groups. Bases for error bars were SD (n = 3), ***p < 0.001.

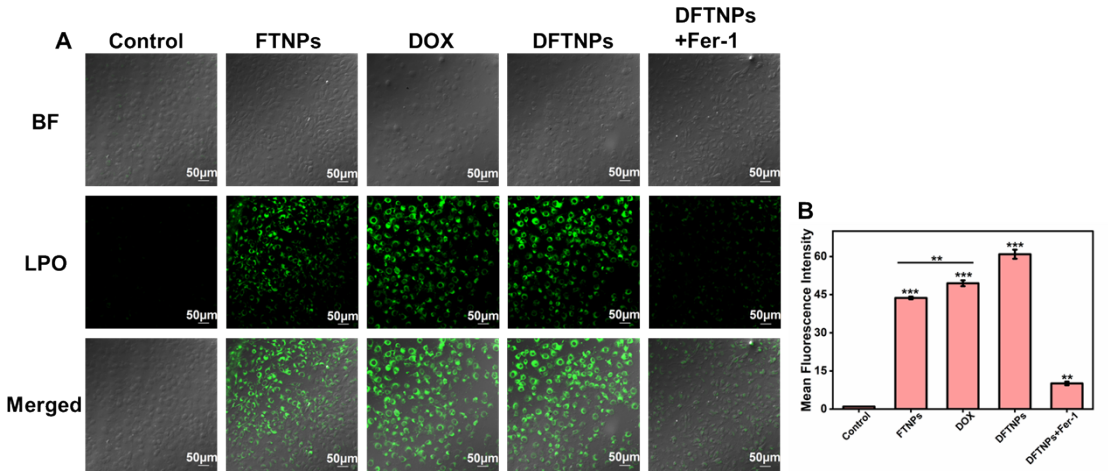

Fig. S14. (A) CLSM images of LPO in MDA-MB-231 cells after different treatments. Scale bar, 50 μm. (B) Quantitative analysis of the mean fluorescence intensity of each group of cells in (A). Bases for error bars were SD ($n = 3$), $^{**}p < 0.01$, $^{***}p < 0.001$.

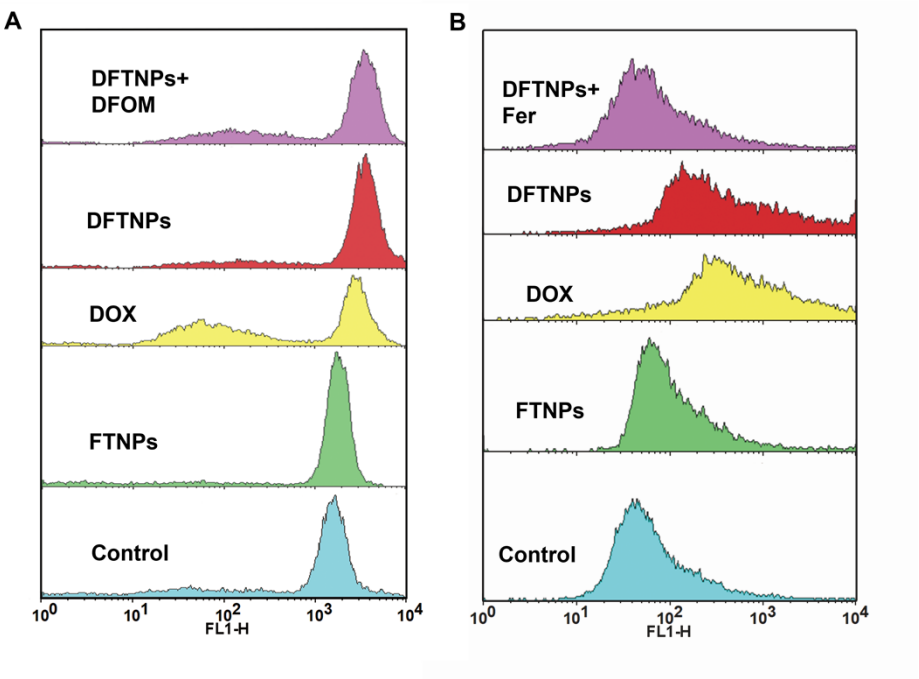

Fig. S15. Flow cytometric quantification of intracellular ROS (A) and LPO (B) levels in MDA-MB-231 cells after different treatments.

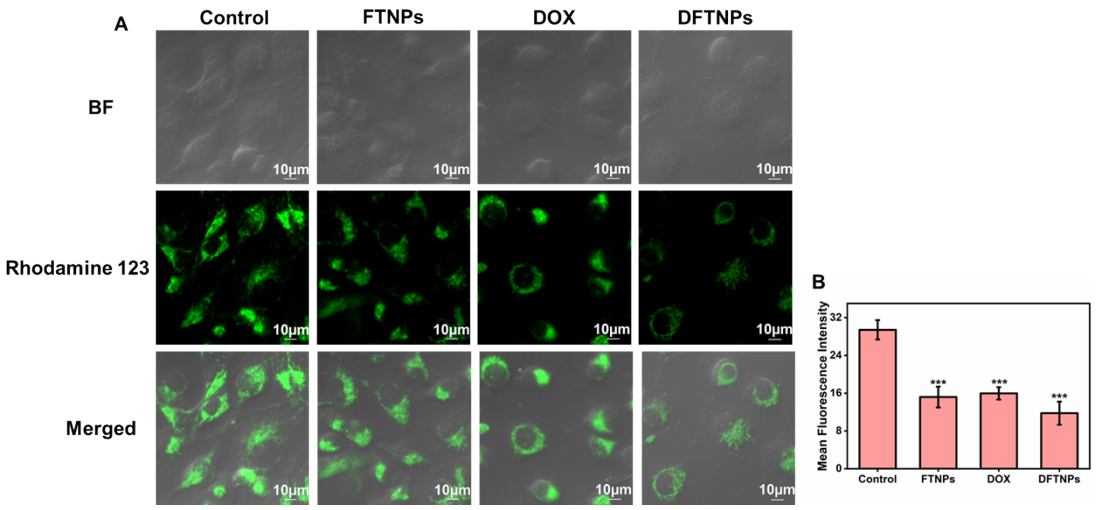

Fig. S16. (A) CLSM images of MDA-MB-231 cells after incubation with different drugs and stained with Rhodamine123. Scale bar, 10 μm. (B) Quantitative analysis of the mean fluorescence intensity of each group in (A). Bases for error bars were SD ($n = 3$), ***$p < 0.001$.

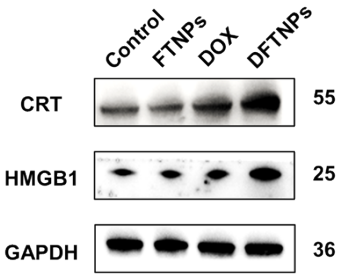

Fig. S17. Determination the expression of CRT and HMGB1 by Western blot in MDA-MB-231 cells.

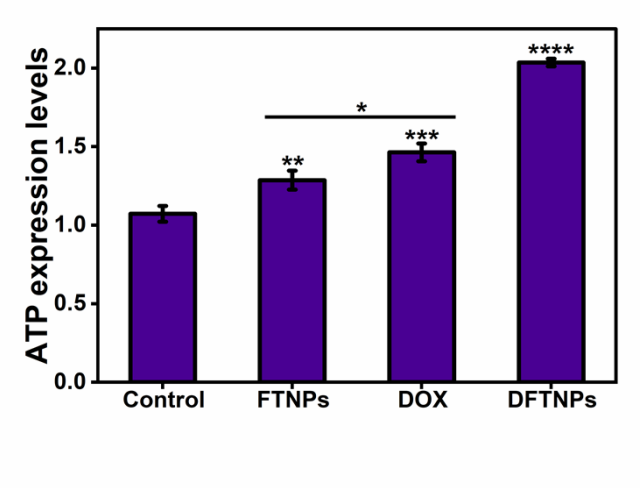

Fig. S18. ATP release from MDA-MB-231 cells after different treatments. The Bases for error bars were SD (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

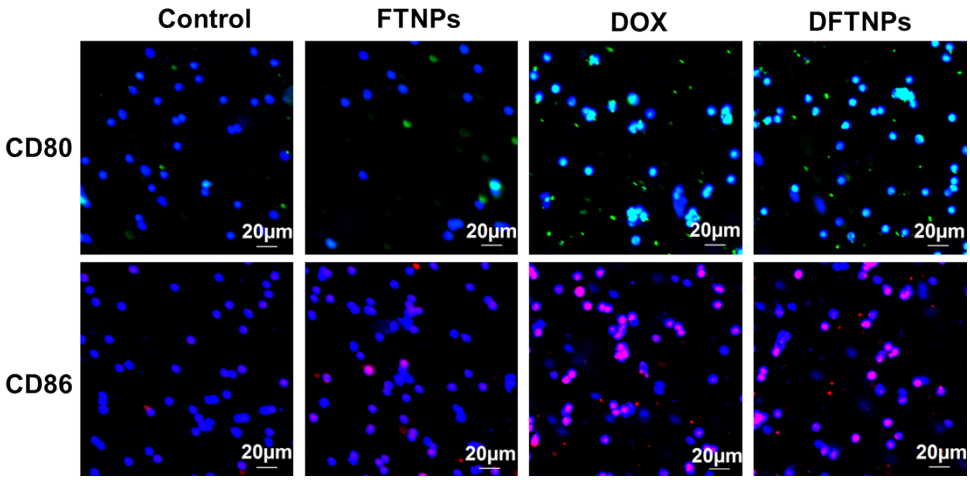

Fig. S19. CLSM analysis of CD80 and CD86 expression in mature DC from BALB/c mice treated with different drugs. Scale bar, 20 μm.

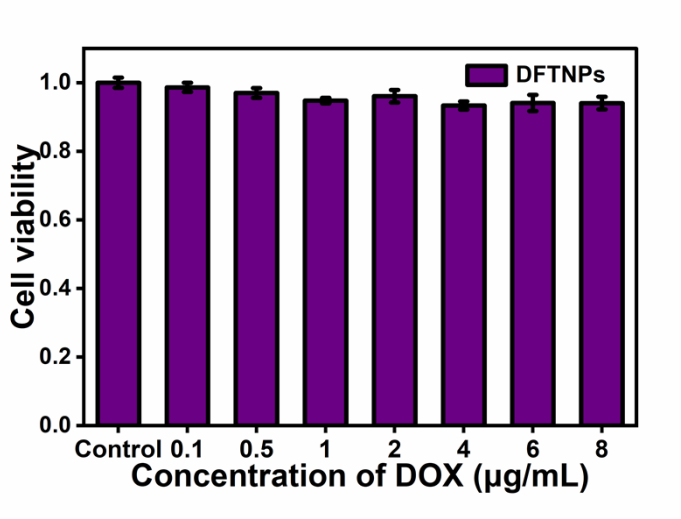

Fig. S20. Cell viability of L929 cells treated with different concentrations of DFTNPs for 24 h. Bases for error bars were SD (n = 3).

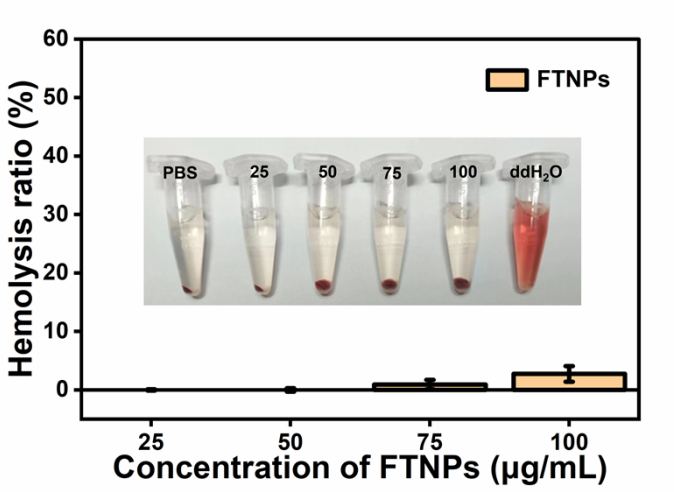

Fig. S21. Hemolytic toxicity of FTNPs at different concentrations. Bases for error bars were SD (n = 3).

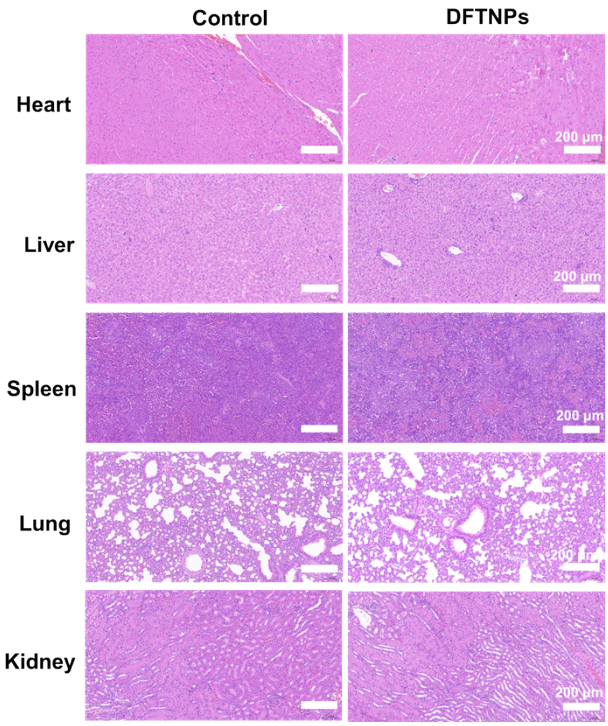

Fig. S22. H&E staining images of major organs sections from BALB/c mice in PBS group and DFTNPs group at the end of treatment. Scale bar, 200 μm.

3. References

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2 L. Yin, J.-J. Duan, X.-W. Bian and S. Yu, *Breast Cancer Res.*, 2020, **22**, 61.