Electronic supplementary information

Self-assembly and self-delivery pure nanodrug of dihydroartemisinin for tumor therapy and mechanism analysis

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Experimental section

Materials

All starting chemicals and solvents were purchased from commercial sources and used without further treatment, unless indicated otherwise. Dihydroartemisinin (DHA) was purchased from Shanghai Aladdin Biochemical Technology Co. Ltd.. TEM images were recorded by JEOL JEM-1011 electron microscope (acceleration voltage of 100 kV). Size and zeta potential were measured by Malvern Zeta Sizer-Nano ZS90 instrument. Annexin V-FITC apoptosis detection kit was purchased from Shanghai Beyotime Biotechnology Co., Ltd. Ultrapure water was prepared from a Milli-Q system (Millipore, USA).

Preparation of DHA NPs

In a typical procedure, 1.136 mg of DHA was first dissolved in 1 mL of tetrahydrofuran (4.0 mM). Then this solution was added dropwise into 10 mL of ultrapure water in ten minutes at room temperature under vigorous stirring, then stirred for several hours to volatilize tetrahydrofuran. At last the crude product was dialyzed against water with a 3500D dialysis bag to removal residual tetrahydrofuran. Finally, DHA NPs were obtained.

The molecular dynamics simulations of DHA NPs self-assembly

The current system was built by PACKMOL [1] program. Firstly, the small molecule was assigned AM1-BCC partial charges using AmberTools 20 package [2]. And then, 30 molecules were randomly posisioned within a cubic box of 30 Å on each side with the mininum distance between atoms being 2.0 Å and the box margin 1.2 Å.

The molecular dynamics (MD) simulations were performed in Yinfo Cloud Computing Platform (YCCP) [3] using AmberTools 20 package [2] with GAFF [4] force fields for the compound. The system was solvated by a truncated octahedron water box using OPC water model with a margin of 10 Å. And then, periodic boundary condition (PBC) was used. Briefly, two 10000 and 10000 steps steepest

descent and conjugate gradient minimizations were performed to remove improper atom contacts. Following initial optimization, the system was then equilibrated during 200 ps NPT and 1 ns NPT ensembles. The temperature was maintained at 300 K using the Berendsen thermostat with 1 ps coupling constant and the pressure at 1 atm using Monte Carlo barostat with 1 ps relaxation time. Finally, 100 ns MD production was performed in NVT ensemble without any restraint.

In vitro stability of NPs

To investigate the in vitro stability, DHA NPs were incubated in water or a solution of PBS (pH 7.4) containing 10% fetal bovine serum (FBS) at temperaure of 37 ^{o}C for different times. The changes of particle size and size distribution were monitored by DLS.

Cellular uptake

Cellular uptakes by HepG2 cells were examined using a confocal laser scanning microscope (CLSM). Cells were seeded in 6-well culture plates (a sterile cover slip was put in each well) at a density of 5×10^4 cells per well and allowed to adhere for 24 h. After that, cells were incubated with DHA NPs (20 μM) for 0.5 h, 2 h, and 4 h at 37^{o}C. Subsequently, the supernatant was carefully removed and the cells were washed three times with PBS. Subsequently, the cells were fixed with 500 μL of 4% formaldehyde in each well for 20 min at room temperature and washed twice with PBS again. Cells were visualized using blue channel for Hoechst 33258 and red channel for NR under a confocal laser scanning microscope.

Cytotoxicity test

The cytotoxicity test was measured *via* MTT assay. HepG2 and MCF-7 cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 10^5 cells/well and incubated in DMEM for 24 h. The medium was then replaced by DHA or DHA NPs, at a final equivalent DHA concentration from 10 to 80 μM for each drug. The incubation was continued for 48 h. Then, 20 μL of MTT solution in PBS with the concentration of 5 mg/mL was added and the plates were incubated for

another 4 h at 37 ºC, followed by removal of the culture medium containing MTT and addition of 150 μL of DMSO to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 min, and the absorbance of formazan product was measured at 490 nm by a microplate reader. Each data point was an average of three independent experiments.

Apoptosis

The apoptosis induced by DHA NPs were evaluated by flow cytometry. HepG2 cells treated with different concentrations of NPs were harvested by centrifugation at 1000 rpm for 5 min, and washed with ice-cold PBS. The cell suspension (100 μL) was centrifuged at 1000 rpm for 5 min. After that, the supernatant was discarded and the pellet was gently resuspended in 195 μL annexin V-FITC binding buffer, and incubated with 5 μL propidium iodide (PI) solution on an ice bath in the dark. After filtration (300 μm), the suspension from each group was analyzed using a flow cytometry.

Total RNA extraction and mRNA library construction

After treatment with DHA NPs, the total RNA was extracted by using Trizol (Invitrogen, Carlsbad, CA, USA) according to manual instruction in HepG2. Finally, total RNA was qualified and quantified by using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA). Then, Oligo(dT)-attached magnetic beads were used to purified mRNA. The purified mRNA was fragmented into small pieces with fragment buffer at appropriate temperature. Then, First-strand cDNA was generated by using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis. Afterwards, A-Tailing Mix and RNA Index Adapters were added by incubating to end repair. The cDNA fragments obtained from previous step were amplified by PCR. The products were purified by Ampure XP Beads, and then dissolved in EB solution. The product was validated on the Agilent Technologies 2100 bioanalyzer for quality control. The double stranded PCR products from previous step were heated denatured and circularized by the splint

oligo sequence to get the final library. The single strand circle DNA (ssCir DNA) was formatted as the final library. The final library was amplified with phi29 to make DNA nanoball (DNB) which had more than 300 copies of one molecular, DNBs were loaded into the patterned nanoarray and single end 50 bases reads were generated on BGIseq500 platform (BGI-Shenzhen, China).

Bioinformatics analysis

The sequencing data was filtered with SOAPnuke (v1.5.2) by removing reads containing sequencing adapter. The clean reads were mapped to the reference genome using HISAT2 (v2.0.4). Bowtie2 (v2.2.5) was applied to align the clean reads to the reference coding gene set，then expression level of gene was calculated by RSEM (v1.2.12). The heatmap was drawn by pheatmap (v1.0.8) according to the gene expression in different samples. Essentially, differential expression analysis was performed using the DESeq2 (v1.4.5) with Q value ≤ 0.05. To take insight to the change of phenotype, GO and KEGG enrichment analysis of annotated different expressed gene was performed by Phyper based on Hypergeometric test. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value ≤ 0.05) by Bonferroni.

Western blot and antibodies

Western blotting was performed using standard methods. After being treated with DHA NPs, the cell pellets were suspended in radio immunoprecipitation assay buffer (150 mM sodium chloride, 50 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 10 mM NaF, 1Mm Na3VO4, 5 mM EDTA, 1 mM EGTA, 5 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mM phenyl methylsulfonyl fluoride, and protease and phosphatase inhibitor for 15 min at 4 ^{o}C and centrifuged at 12000 rpm 20 min at 4 ^{o}C. Protein concentration of the supernatants was determined using a BCA protein Assay Kit (Beyotime Biotechnology, China). Equal amounts (50 μg) of the proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis gels and transferred to PVDF membranes

(Millipore) by using a Bio-Rad Trans-blot instrument. Membranes were blocked in 5% milk for 1 h at room temperature following incubation with the indicated primary antibody overnight at 4 °C, washed 3 times with TBS-T buffer, incubated with secondary antibody IgG-HRP at 1:20000 dilutionsin TBS-T buffer (Cat. #A11034 for anti-rabbit; Cat. #A-32723 for anti-mouse by Invitrogen). After washing 3 times with TBS-T buffer, the membrane was developed with ECL substrate (Thermo Scientific) and the signal was detected by a BIO-RAD Fluorescent Imager, following quantification by Image Lab software.

The following antibodies were used in this study, all diluted in in TBS-T including 3% milk. Antibodies directed against, β-actin (CL594-6600), Bax (60267-1-Ig), Bcl-2 (60178-1-Ig), p53 (60283-2-Ig) were obtained from proteintech (1:2000 dilution). Cleaved Caspase-3 (ab32042), Cleaved Caspase-9 (ab2324), Cytochrome C (ab13575), were obtained from Abcam (1:5000 dilution). Noxa (PA5-19977) and Puma (PA5-34755) were obtained from Thermo Scientific (1:1000 dilution).

In vivo antitumor test and safety evaluation

All the experimental procedures to mouse described herein have gained approval from the Ethics Committee of Jilin Medical University and carried out corresponding to the regulation, principles, and guidelines of Chinese law concerning the protection of animal life. Kunming (KM) female mice were obtained from Jilin University and maintained under required conditions. The H22 xenograft tumor models were established by injecting H22 hepatocellular carcinoma cells into the left infra-axillary dermis of the mice. When the tumor grew to a size of ~100 mm^3, H22 bearing Kunming mice were randomly divided into three groups with 5 mice in each group: PBS, free DHA, and DHA NPs. Mice were administered PBS, free DHA, and DHA NPs with the same dosage of 15 mg/kg DHA *via* tail vein injection once every other two days, respectively, and the tumor volume and body weight were measured every three days in 21 days. After 21 days of observation and measurement, the mice of four groups were sacrificed and the tumors were excised to intuitionally evaluate the tumor inhibition. At last, main organs (heart, liver, spleen, lung, kidney) and tumors were

collected, fixed in 4% paraformaldehyde solution, and then embedded in paraffin, sliced and stained with hematoxylin and eosin (H&E) to evaluate potential toxicity for main organs and apoptosis degree for cancer cells. Ki-67 is a proliferation marker reflecting the degree of proliferation activity of tumor cells. The cell apoptosis was evaluated by terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) staining using an *in situ* Cell Death Detection Kit following the manufacturer's instruction.

Results

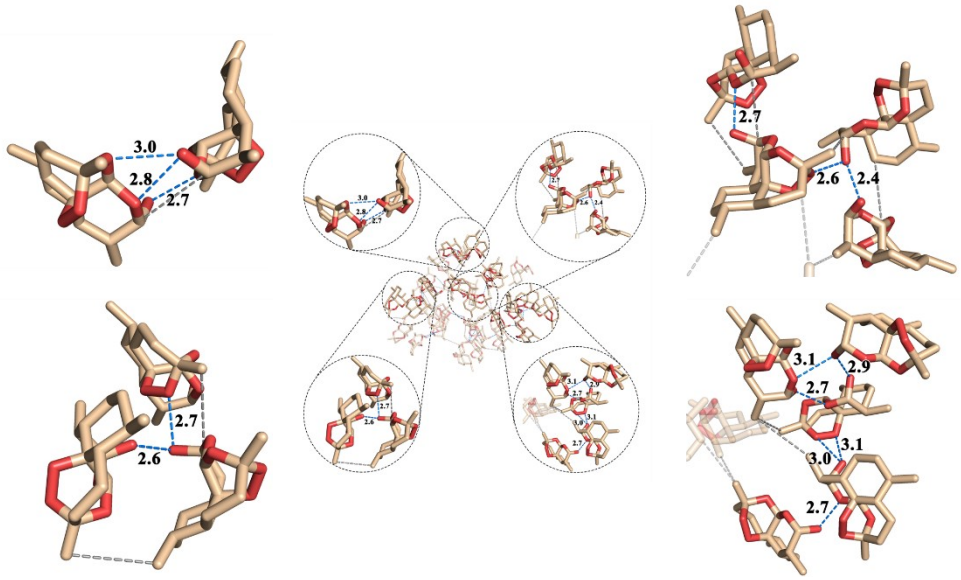

Fig. S1. The molecular dynamics simulations of DHA NPs self-assembly. The DHA self-assembly into DHA NPs were mainly via hydrophobic interactions (gray sticks) and hydrogen bond interactions (blue sticks).

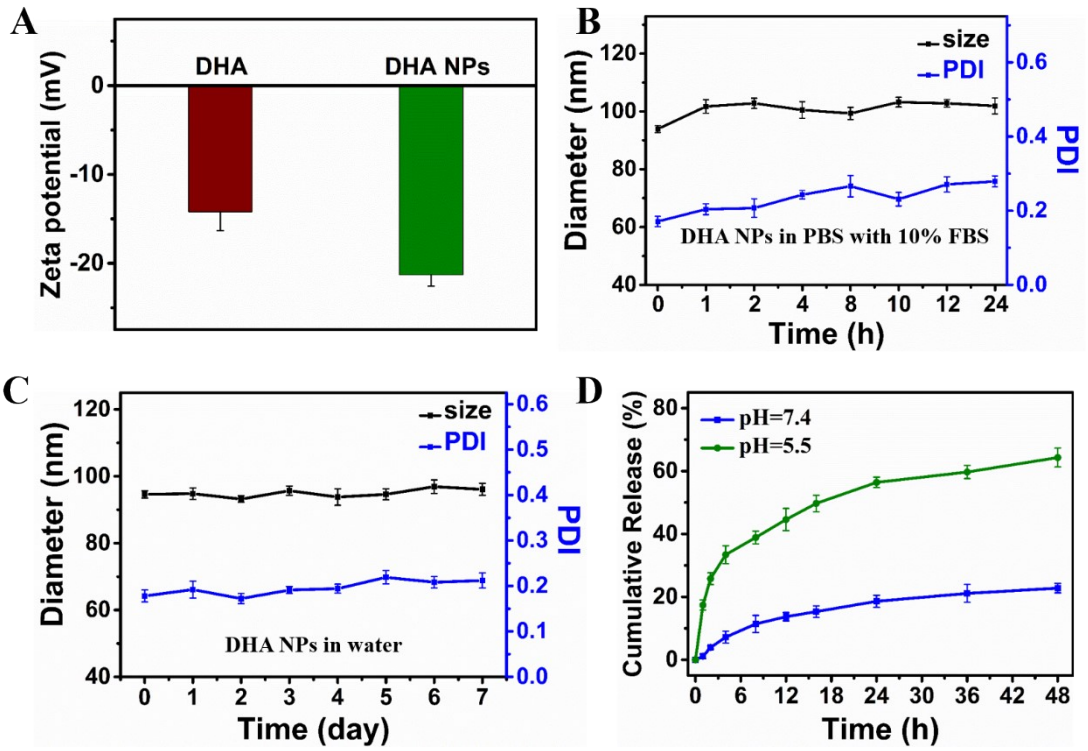

Fig. S2. (A) Zeta potentials of DHA and DHA NPs. **(B)** Changes of hydrodynamic diameter and PDI of DHA NPs stored in PBS with FBS (10%) for 24 h. Data are expressed as mean ± SD (n = 3). **(C)** Changes of hydrodynamic diameter and PDI of DHA NPs stored in water for 7 days measured by DLS. **(D)** Cumulative drug release profiles of DHA NPs in different PBS solutions. Data are expressed as mean ± SD (n = 3).

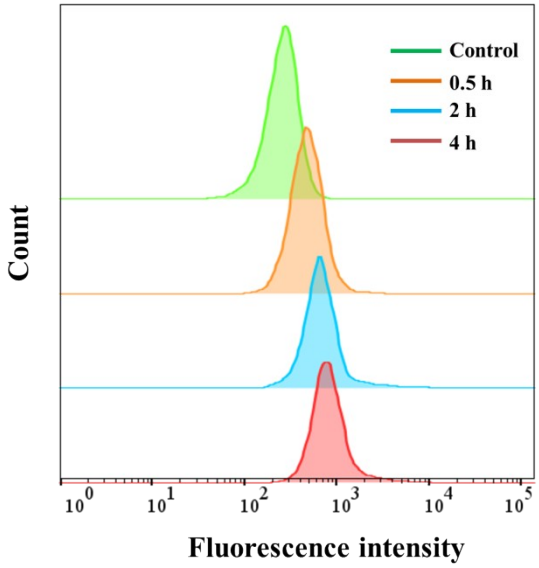

Fig. S3. FCM analysis of cells incubated with DHA NPs at 37 °C for 0.5, 2, and 4 h.

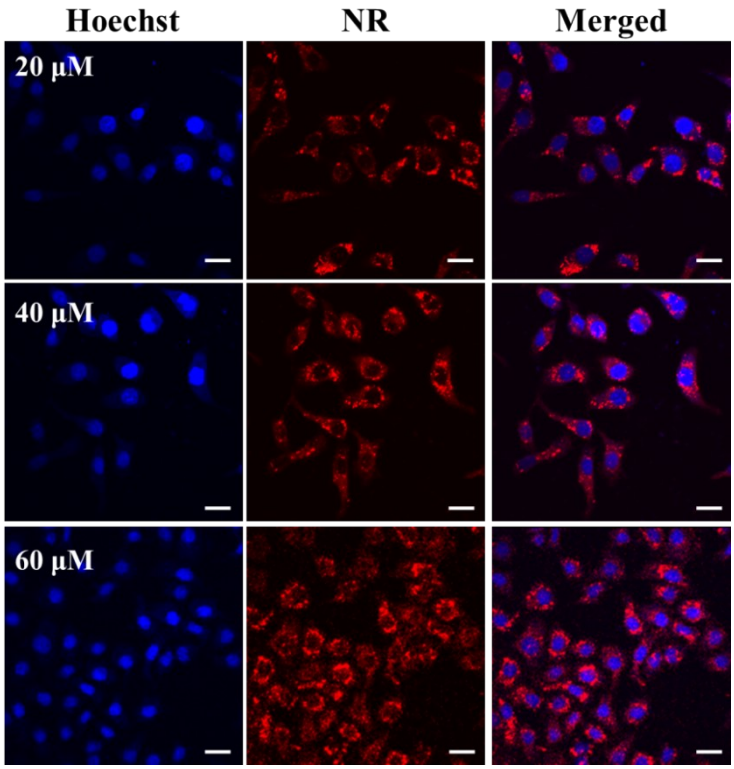

Fig. S4. CLSM images of HepG2 cells incubated with different concentrations of DHA NPs for 4 h at 37 °C. All scale bars are 20 μm.

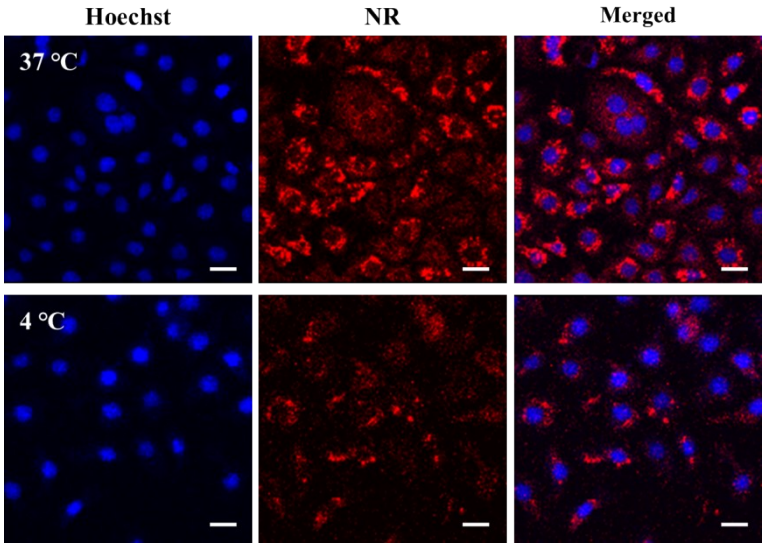

Fig. S5. CLSM images of HepG2 cells incubated with DHA NPs at 37 °C and 4 °C for 4 h. All scale bars are 20 μm.

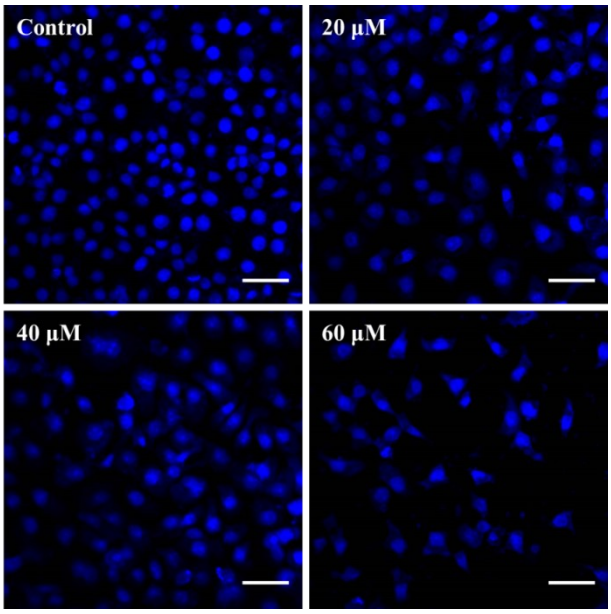

Fig. S6. Morphological apoptosis by staining with Hoechst 33258 in HepG2 cells treated with different concentrations of DHA NPs. All scale bars are 20 μm.

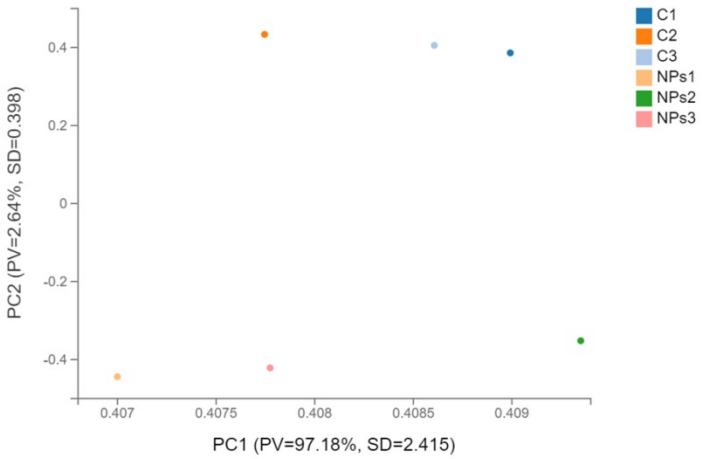

Fig. S7. Principal component analysis (PCA) of HepG2 cells based on untreated control group (C) and DHA NPs treatment group (NPs).

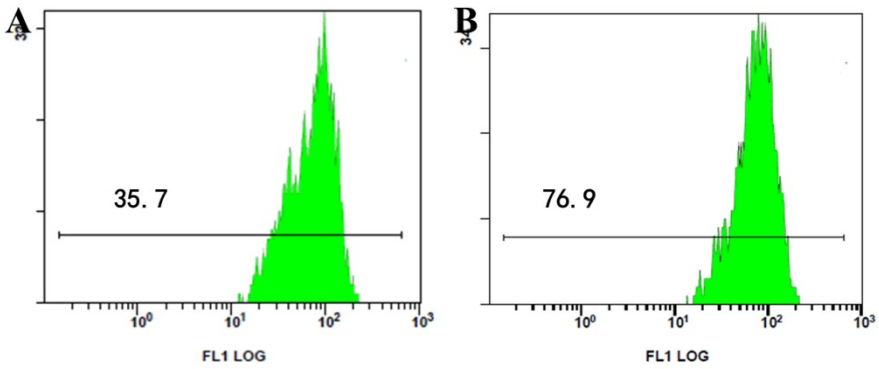

Fig. S8. FCM analysis of ROS generation of HepG2 cells in A) control group and B) DHA NPs treatment group.

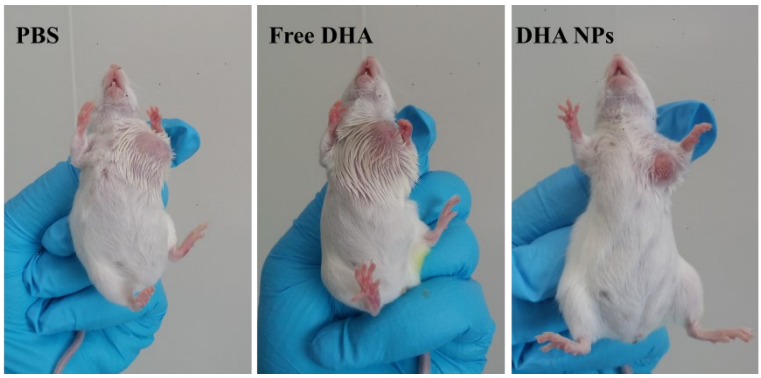

Fig. S9. Representative photographs of mice in different groups after various treatments.

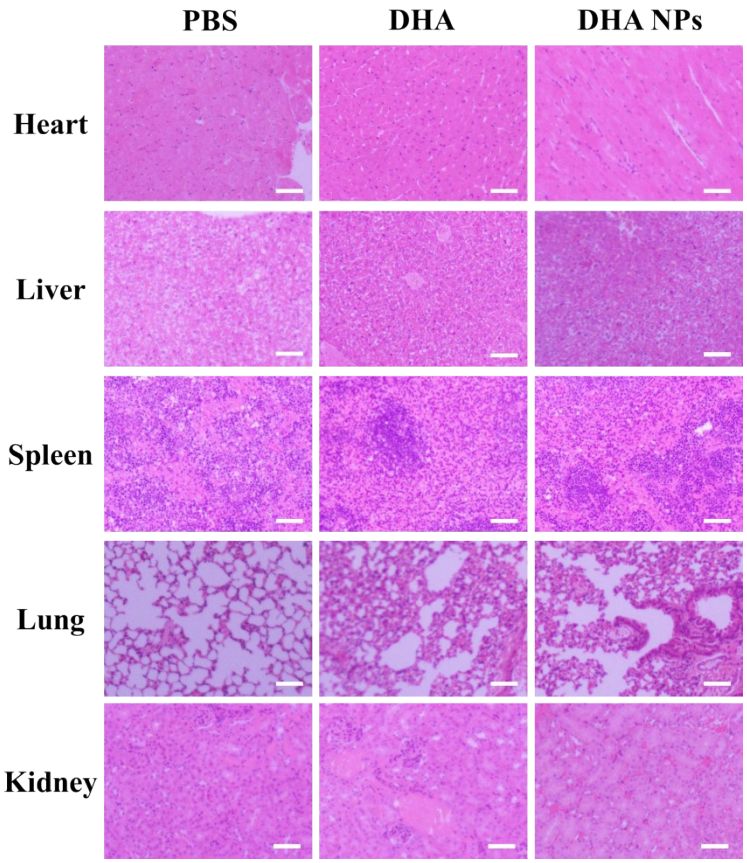

Fig. S10. H&E staining images of the major organs (heart, liver, spleen, lung and kidney) of mice from each group at day 12. Scale bars: 100 μm.

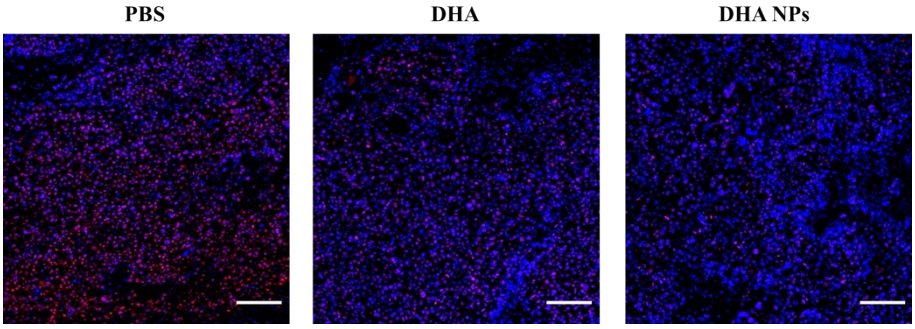

Fig. S11. Ki67 staining images of tumor slices in different groups. Scale bars, 100 μm.

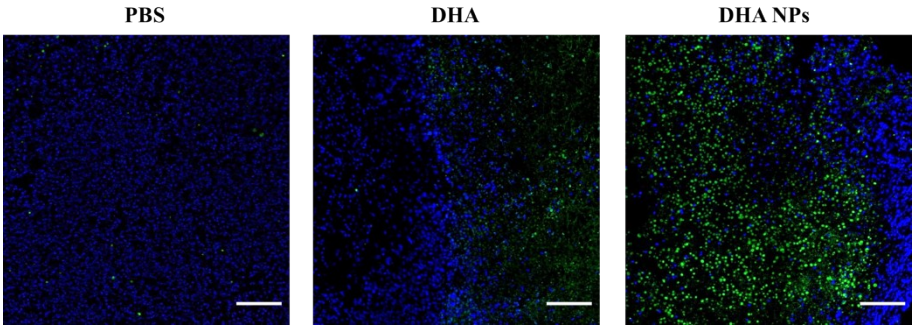

Fig. S12. TUNEL staining images of tumor slices in different groups. Scale bars, 100 μm.

Reference

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