# Electronic supplementary information

# Supramolecular Hybrids of Carbon Dots with Dihydroartemisinin for Enhanced Anticancer Activity and Mechanism Analysis

Yawei Li , Nianqiu Shi, Wei Zhang, Hong Zhang, Yu Song, Wenhe Zhu*, and Xianmin Feng*

Jilin Medical University, Jilin 132013, P. R. China

* Corresponding author E-mail: jlmpczwh@163.com

## Experimental Section

**Materials and Characterization**

All starting chemicals and solvents were purchased from commercial sources and used without further treatment, unless indicated otherwise. UV-vis absorption spectra were recorded via a Shimadzu UV-2450 UV-vis scanning spectrophotometer. Fluorescence emission spectra were conducted on a LS-55 fluorophotometer. 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.

**Preparation of CDs and CDs-DHA**

For a typical synthesis of CDs, D-glucose (2.5 mmol) and L-glutamic acid (2.5 mmol) were loaded into a beaker and then 1 M sodium hydrate (3.0 mL) was added. The transparent solution was heated to 125 $^{o}$C and kept for 30 min, then heated to 200 $^{o}$C and maintained for 20 min. The final reaction products were completely solubilized, and then placed in a dialysis bag (Mw cutoff: 3.5 kDa) and dialyzed against water to remove small molecules for 1 d. The resulting product was freeze-dried to obtain brown solid.

For a typical synthesis of CDs-DHA, 30 mg of CDs was dissolved in 10 mL of deionized water; on the other hand, 36 mg of DHA (the weight ratio of DHA to CDs is 1.2:1) was dissolved in 3 mL of tetrahydrofuran (THF); thereafter, the DHA solution was slowly dropped into the aqueous solution of CDs, and the mixture of CDs and DHA were stirred overnight at room temperature to form the hybrids of CDs-DHA. Then the reaction solution was centrifuged at 5000 rpm for 5 min in order to remove the unassembled hydrophobic DHA. The obtained supernatant was placed in a dialysis bag (Mw cutoff: 3.5 kDa) and dialyzed against deionized water for 24 h to remove THF. Finally, CDs-DHA were obtained.

**In vitro drug release study**

The freeze-dried CDs-DHA nanoparticles were dissolved in PBS containing 0.5% Tween 80 (pH 7.4 and 5.5) at a concentration of 1 mg/mL. The above mixture was transferred into a dialysis bag with a molecular weight cutoff of 3500 Da. The bag was then immersed into a container with 20 mL of PBS containing 0.5% Tween 80 at the same pH value as that inside the bag. The entire system was kept in a vapor-bathing constant temperature vibrator at 37 °C under continuously shaking at 100 rpm. At selected time intervals, 3 mL of the external buffer was withdrawn for UV-vis analysis and replaced with the same amount of fresh buffer solution. The released amount of DHA was determined from the absorbance at 290 nm with the help of a calibration curve of DHA in the same buffer. Then the accumulative weight and relative percentage of the released DHA were calculated as a function of incubation time.

**Cell culture**

The human hepatocellular carcinoma HepG2, SMMC-7721 and BEL-7404 cells and normal hepatocytes (HL-7702) cells were routinely grown in DMEM medium and cultured in medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 $^{o}$C under 5% $CO_2$.

**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 $5\times10^4$ cells per well and allowed to adhere for 24 h. After that, the cells were treated with CDs-DHA NPs (20 μM) for 0.5 h at 37 $^{o}$C. Thereafter cells were incubated for additional 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 green channel for CDs-DHA under a confocal laser scanning microscope (Carl Zeiss LSM 700).

**Cytotoxicity Test**

The cytotoxicity test was measured via MTT assay. HepG2, SMMC-7721 or BEL-7404 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 CDs-DHA, at a final equivalent DHA concentration from 10 to 100 μM for each drug. The incubation was continued for 24 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 $^{o}$C, 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 and necrosis induced by CDs-DHA NPs were evaluated by flow cytometry. HepG2 cells treated with different concentrations of CDs-DHA 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.

**Measurements of glucose uptake levels, lactate production**

Logarithmic growth HepG2 cells were seeded in 6-well plates with a density of $1\times10^4$ cells/well. After treatment with CDs-DHA for 24 h, the cell culture media was collected for detecting the glucose uptake and lactate production. The content of glucose in the collected culture media was detected immediately following the manufacturer's instructions of Glucose Assay Kit. For assessment of lactate production, the collected culture media was diluted 1:50 by using lactate assay buffer. The amount of lactate present in the media was then estimated using the Lactate Assay Kit (sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

**Western blot and antibodies**

Western blotting was performed using standard methods. After CDs-DHA treatment, $5\times10^5$ HepG2 cells were harvested and lysed in PIPA buffer to extract the total protein. The extract total protein content was determined by Bio-Rad protein assay kit and 30 μg protein/lane was loaded on SDS-PAGE. After SDS-PAGE, the separated proteins were transferred to PVDF membranes. Non-specific binding was blocked by incubating nitrocellulose

membranes in 5% no-fat dry milk in TBS/0.1% Tween for 120 min. After blocking, the blots were incubated with primary antibody at 4°C overnight. Then, the appropriate secondary antibody was added and incubation for 1 h at room temperature. Immunoreactive protein bands were detected by chemiluminescence using enhanced chemilumunescence reagents (ECL).

**UALCAN**

UALCAN (http://ualcan.path.uab.edu/index.html) was used to analyse TCGA data. UALCAN was used to examine gene expression in tumor and normal samples and changes in factors associated with various tumours, such as tumour stage, tumour classification, race, weight, and other clinicopathological features. The expression of PKM2 and the effect of PKM2 gene expression on patient survival conditions were identified in UALCAN.

**Establishment of stable PKM2 overexpression in HepG2 cells**

For transfection, HepG2 cells were plated at a concentration of $3 \times 10^5$ cells/well in 6-well plates. The constructed plasmid pcDNA 3.1-PKM2 and plasmid pcDNA 3.1 were transfected into the HepG2 cells by using liposome transfection methods, according to the instructions of manufacturer. 48 h after transfection, cells were passaged and were cultured in DMEM medium containing 10% fetal bovine serum and 500 ug/ml G418. Media were exchanged every 3-4 days. When cell death discontinued and a few cells underwent division and proliferation began to increase, 200 μg/ml G418 was used to maintain screening. Cells were digested in the original cell culture bottles when positive cell clones were grown to a certain number. Cells were processed after reaching 100% confluence. One week later， the cells were used in the experiments.

**Quantitative reverse transcription PCR (qRT-PCR)**

The expression of PKM2 was characterized using total RNA extracted from CDs-DHA treated and untreated HepG2 cells. Real-time RT-PCR was performed on RNA samples using primers are as follows: PKM2 forward: 5′-GCCATAATCGTCCTCACCAAGT-3′, reverse 5′-GCACGTGGGCGGTATCTG-3′; β-actin forward 5′- ACTCTTCCAGCCTTC CTTC-3′, reverse 5′- ATCTCCTTCTGCATCCTGTC-3′. The iScript One-Step RT-PCR Kit with SYBR Green and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All values were normalized to β-actin and then standardized to the control condition. Error bars represent S.E.M., and the statistical significance was assessed using the Student's t-test. After completion of the RT-PCR, Ct values were obtained from the ABI 7500 fast v2.0.1 software. The $^{\Delta\Delta}$Ct method was used to represent mRNA fold change.

**In vivo antitumor test**

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 four groups with 3 mice in each group: Control, pure CDs, free DHA, CDs-DHA NPs. Mice were administered PBS, CDs, free DHA or CDs-DHA NPs with the same dosage of 5 mg /kg DHA via tail vein injection once every 2 days, respectively, and the tumor volume and body weight were measured every other day in 12 days. After 12 days of observation and measurement, the mice of four groups were sacrificed and the tumors were excised to intuitionally evaluate the tumor inhibition.

**Statistical Analysis**

All experiments were performed at least three times and all results were expressed as mean ± standard deviation (SD). Student's t-test was used to determine the statistical difference between various experimental and control groups. Significant differences between the groups are indicated by * for $p < 0.05$, ** for $p < 0.01$, and *** for $p <$

0.001, respectively.

## Results

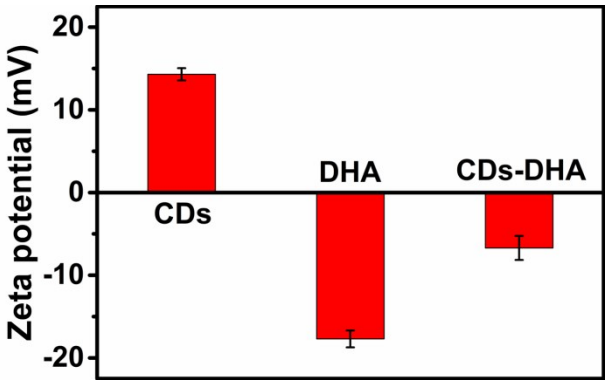


**Fig. S1** Zeta potential of CDs, DHA and CDs-DHA.

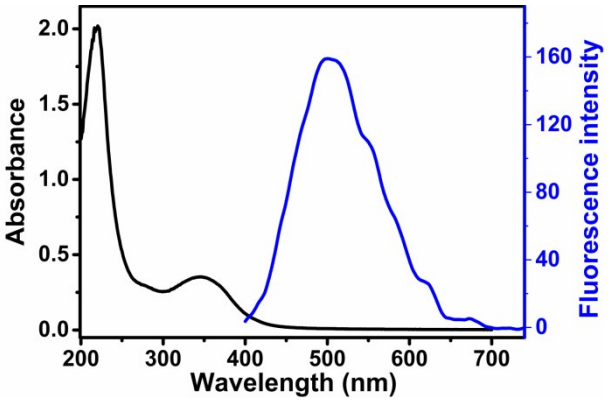


**Fig. S2** Absorption and PL (excited at 360 nm) spectra of CDs.

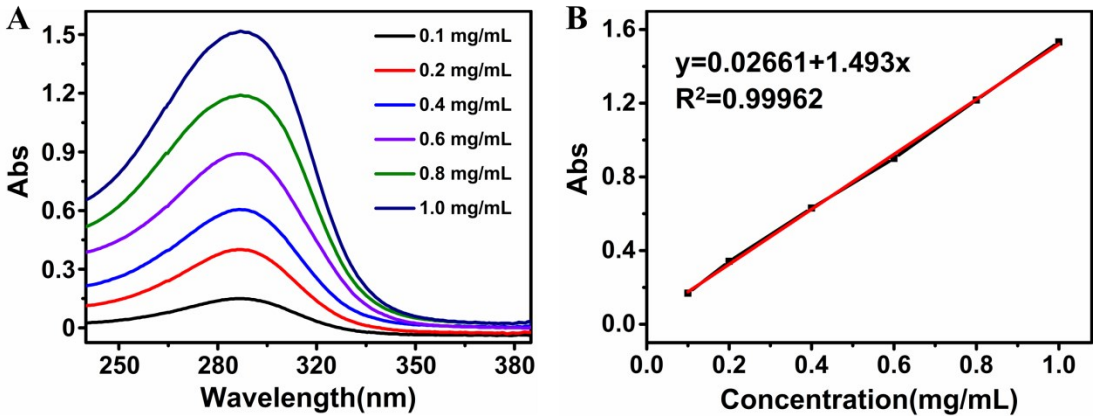


**Fig. S3** The quantification of DHA loading by UV-vis spectra. (A) UV-vis absorption curves of DHA solutions with different concentrations. (B) The standard curve for absorbance values at 290 nm.

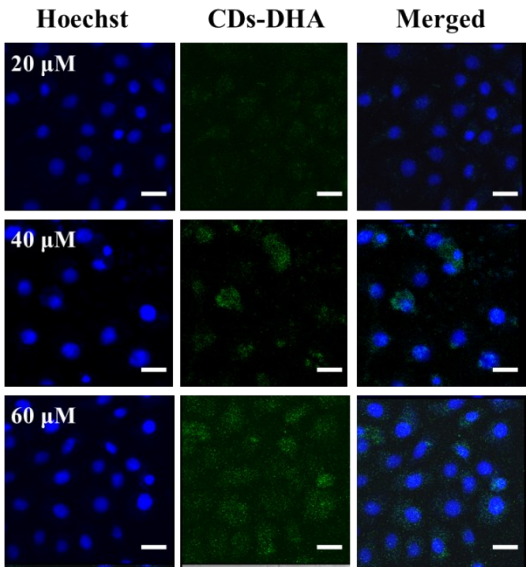


**Fig. S4** CLSM images of HepG2 cells incubated with different concentrations of CDs-DHA for 4 h at 37 °C. The nuclei were stained by Hoechst 33258. All scale bars are 20 μm.

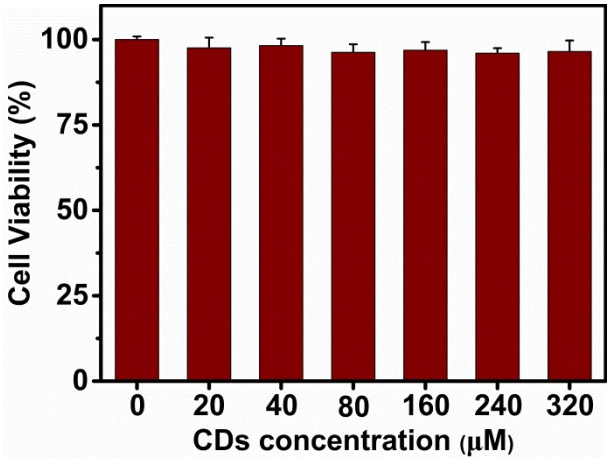


**Fig. S5** Cell viabilities of HL-7702 cells after incubation with various levels of CDs for 24 h.

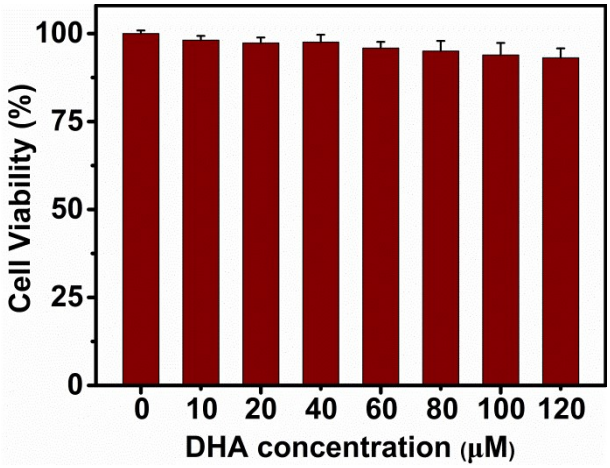


**Fig. S6** Cell viabilities of HL-7702 cells after incubation with various levels of CDs-DHA for 24 h.

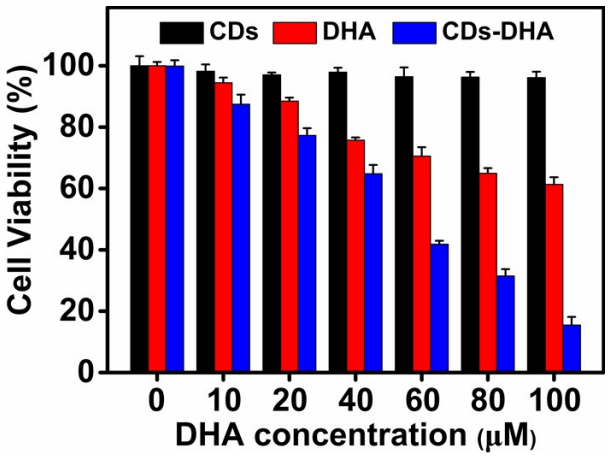


**Fig. S7** *In vitro* cytotoxicities of CDs, free DHA and CDs-DHA against SMMC-7721 cells at different concentrations after 24 h. Data represent mean values ± standard deviation, *n* = 3.

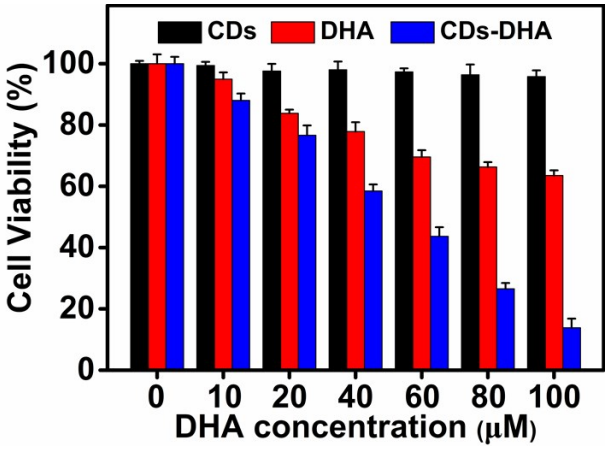


**Fig. S8** *In vitro* cytotoxicities of CDs, free DHA and CDs-DHA against SMMC-7404 cells at different concentrations after 24 h. Data represent mean values ± standard deviation, *n* = 3.

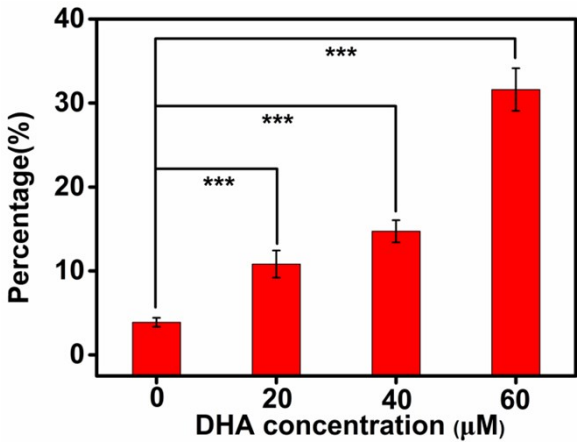


**Fig. S9** The relative quantitative analysis of early apoptosis in HepG2 cells treated with different concentrations of CDs-DHA. Statistical significance: $^{**}p < 0.01$, and $^{***}p < 0.001$.

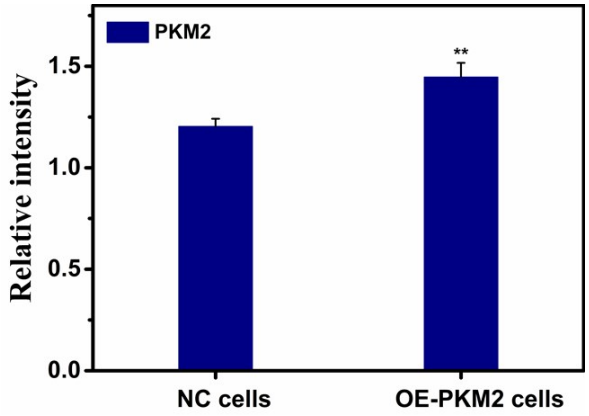


**Fig. S10** The expression of PKM2 in negative control (NC) cells and in overexpressing PKM2 (OE-PKM2) cells were examined by western blot. Densitometric values were normalized by β-actin and expressed as mean ± SD, n = 3.

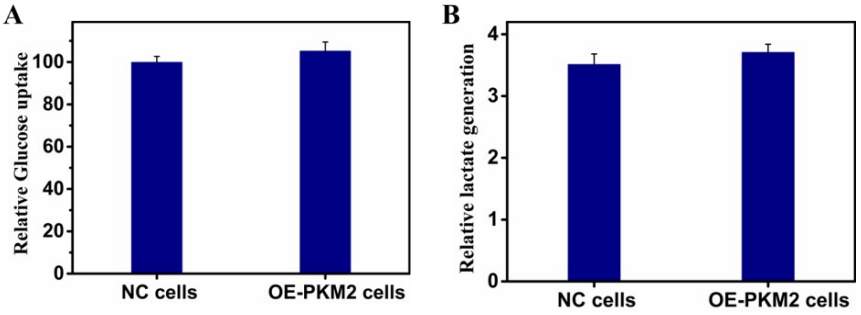


**Fig. S11** (A) Glucose uptake and (B) Lactate product in NC cells and in OE-PKM2 cells.