Supporting information

Demethoxycurcumin-Carrying Chitosan-Antibody Core-Shell Nanoparticles with Multi-therapeutic Efficacy toward Malignant A549 Lung Tumor-From In-Vitro Characterization to In-Vivo Evaluation

Wei-Ting Huang[a], Mikael Larsson[a,b], Yen-Ren Wang[a], Shih-Hwa Chiou[c], Hui-Yi Lin[d] and Dean-Mo Liu[a*]

[a] Nano-Bioengineering Lab, Department of Material Science and Engineering, BioICT Consortium, National Chiao Tung University, Hsinchu 301, Taiwan

[b] Ian Wark Research Institute, University of South Australia, Mawson Lakes Campus, Mawson Lakes, SA 5095, Australia

[c] Institute of Pharmacology, National Yang-Ming University & Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan

[d] School of Pharmacy, China Medical University, Taichung, Taiwan

*Corresponding author:
Phone: 886-3-571 2121 ext. 55391
Fax: +886 3 572 4727
Email: deanmo.liu@gmail.com

Experimental

2.1. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4,6-diamidino-2-phenylindole (DAPI), 2-propanol, sodium hydroxide, chloroacetic acid, and phosphate buffered saline (PBS) were purchased from Sigma Aldrich. CHC was purchased from Advanced Delivery Technologies, Inc. PSVue®480 was purchased from Molecular Targeting Technologies, Inc. Demethoxycurcumin was a courteous gift from China Medical University (Taiwan). The antibody against extracellular signal-regulated kinase (ERK) 1/2, β-actin, EGFR, HIF-1α, P-gp, horseradish peroxidase-conjugated mouse anti-human IgG and horseradish peroxidase-conjugated rabbit anti-human IgG antibody were purchased from Genetex. All other chemical reagents in the study were analytical grade and used as received without further purification.

Western blot analysis

A549, hypoxic A549 and hypoxic A549 treated with 10 μg/mL DMC for 24 h were cultured in 100 mm^2 dishes. After the treatment the cells were lysed and the proteins collected

for Western blot analysis. All protein samples were quantified through Bradford assay. 8% and 10% one-dimensional sodium dodecyl sulfate polyacrylamide gel (SDS-page) was prepared and each protein sample was loaded at 100 μg/well for the different proteins targets (β-actin, EGFR, P-gp, ERK and HIF-1α). After gel electrophoresis, the gel was transferred on nitrocellulose filter membrane (Millipore, USA) and immunoblotting was performed separately using EGFR antibody (1: 2000, Genetex), P-gp antibody (1:70, Genetex), ERK antibody (1:10000, Abcam), HIF-1α antibody (1:200, Genetex) and β-actin antibody (1:5000, Abcam). Before introduction of 2nd antibody, the membrane was washed in PBS three times to remove non-specifically bond 1st antibody. The membrane was then incubated with anti-mouse or anti-rabbit antibody with horseradish peroxidase and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyper film and ECL reagents (Amersham International PLC, Buckinghamshire, U.K.). The results were quantified using the Image J software and were expressed as relative intensity compared with the control β-actin.

Detailed version of: 2.3. Preparation and characterization of CHC/DMC/anti-EGFR nanoparticles

DMC loaded self-assembled CHC nanoparticles were prepared as follows; 5 mg CHC in 8 ml H2O was mixed with 2 mg of DMC in 2 ml methanol and stirred for 12 h at RT. Subsequently, 1 μg/mL EGFR antibody and 0.1% EDC reagent were added to the solution, followed by continued mixing for 3 h. Non loaded DMC was removed by centrifugation at 12000 rpm for 30 min, discarding the pellet. The methanol was then removed using a rotary evaporator. The loading efficiency was determined by analyzing the amount of non-loaded DMC by high performance liquid chromatography (HPLC) with a C18 column, monitoring absorbance at 425 nm.

The size of bare CHC, CHC/DMC and CHC/DMC-anti-EGFR nanoparticles suspended in PBS buffer was measured through dynamic light scatter (DLS, Beckman Coulter, Inc., USA). The morphology of bare CHC was examined by scanning electron microscope (SEM, JEOL 6700, Japan). The SEM sample was prepared after CHC nanoparticles had completed self-assembly by adding a drop (0.05% wt/v) onto a silicon wafer, followed by drying. The sample was subsequently sputtered with platinum before the SEM analysis for better image quality. The structure of CHC/DMC and CHC/DMC-anti-EGFR nanoparticles was analyzed through transmission electron microscope (TEM, JEOL 2100, Japan). TEM samples were prepared immediately after CHC/DMC and CHC/DMC-anti-EGFR nanoparticles had been synthesized. Both samples (1 mg/mL) were put on 200-mesh copper grids coated with carbon. For the CHC/DMC nanoparticle sample, negative staining was used to observe DMC in CHC carrier.

2.3.1. In vitro release study

After the preparation, as given above, the nanocarriers were lyophilized, added to 10 mL of PBS, pH 7.4 (final concentrations of CHC and DMC were 5 mg/ml and 2 mg/ml, respectively) and stirred at 37°C. Since DMC released from the CHC carriers has extremely low water solubility, the DMC released at the different times was separated by centrifugation at 5000 rpm

for 30 min. The DMC was subsequently dissolved in 400 μL methanol, and 20 μL of the solution was used in HPLC analysis to determine the DMC concentration. The percentage of DMC released at the different times was calculated from the following equation:

released DMC (%) = [(released DMC from CHC nanoparticles)/(total amount of DMC in CHC nanoparticles)] X100；

The release profiles were analyzed using the Korsmeyer-Peppas model, fitting the variables to data [1].

Detailed version of: 2.5. The interaction between cells and nanoparticles

2.5.1. Cellular uptake

A flow cytometer was employed to determine the internalization of CHC/PSVue nanoparticles and CHC/PSVue-anti-EGFR nanoparticles with A549. A549 were plated at $1x10^6$ cells in a 100 mm dish and allowed to attach for 24 h. The culture medium was discarded, and the cells were washed with PBS. To examine the effect of incubating time, the cells were incubated with both kinds of nanoparticles for predetermined time periods at 37°C. The amounts of CHC/PSVue nanoparticles were equal in dose to the CHC/PSVue-anti-EGFR nanoparticles. After the cellular uptake treatment, the A549 were washed twice with PBS and then collected by trypsinization. Then, A549 were centrifuged, and dehydrated with 70% ethanol overnight at -20°C, followed by resuspension in PBS. To avoid cell aggregation, the cell solutions were filtered through a nylon membrane (BD Biosciences, USA). A minimum of ten thousand dead cells were analyzed. The cellular uptake of nanoparticles was determined by BD FACS Calibur flow cytometry, and the fluorecense intensity was quantified by CellQuest software CellQuest Pro software (BD Biosciences, USA).

2.5.2. Intracellular drug distribution

To explore *in vitro* drug distribution of free DMC, CHC/DMC and CHC/DMC-anti-EGFR nanoparticles after their internalization within A549, the cells were seeded on 24-well culture plates with a seeding density of $1x10^5$ cells/ well. After the cells had attached, the wells were carefully washed with PBS, and free DMC or nanoparticles at a concentration of 1 μg/mL were added along with the media in triplicate to the wells, after incubation for different times (1, 2, 4, 12, and 24 h) the cells were processed for quantification of DMC by HPLC. Sample preparation for intracellular drug distribution involved pelletization of treated cells at 12,000 rpm for 5 min, drying, and lysis of the pellet in methanol followed by probe sonication at high amplitude for 5 min so that DMC could be extracted into the methanol fraction. The lysate was centrifuged at 10,000 rpm for 5 min, and HPLC was used to determine the amount of intracellular DMC at the different times.

2.5.3. Cell TEM sample preparation

To observe the distribution of CHC/DMC-anit-EGFR nanoparticles in A549, TEM analysis was used. A549 cells were incubated with nanoparticles for 12 h and then collected through trypsinization. The cell pellet with nanoparticles was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer and stained with 1% OsO_4 in 0.1 M phosphate buffer for hours. After

staining, the sample was dehydrated through increasing percentage ethanol and 100% acetone. Finally, the sample was embedded in Spurr's resin at 65ºC for 48 h. For TEM observation, sample was sectioned with a diamond knife into 80 nm thick slices and put on 200-mesh copper grids for analysis.

Results

DLS and zeta-potential measurements of: 3.1. Characteristics of CHC-DMC/Anti-EGFR core-shell nanoparticles

The size (number average) of the nanoparticles increased from about 50 nm for bare CHC to 130 nm and 180 nm after DMC loading and surface modification, respectively. That is, the loading with DMC increased the size by a factor of about 2.5, which corresponded to a factor 16 increase in particle volume. The surface modification with anti-EGFR antibody resulted in a further 60 nm size increase, corresponding to the formation of a 30 nm antibody shell. The zeta potential of bare CHC nanoparticle was -22.5±0.91 mV, while for CHC/DMC and CHC/DMC-anti-EGFR nanoparticle it was -15.0±3.5 mV and -5.1±0.8 mV., respectively.

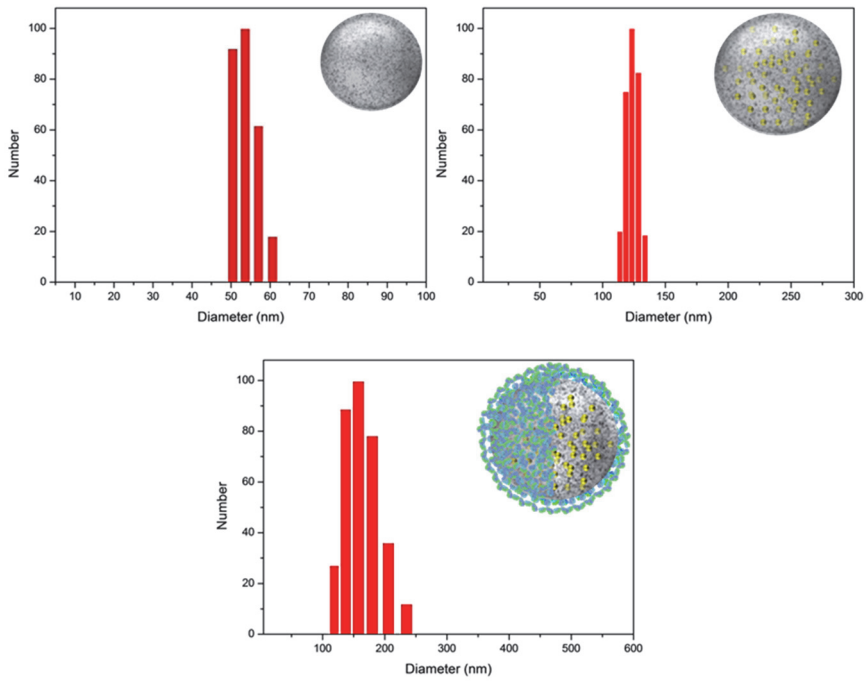

Fig. S1. *Size characteristics of CHC nanoparticles. Dynamic light scattering number distribution of nanoparticle sizes for CHC (left), CHC/DMC (right) and CHC/DMC-anti-EGRF (middle).*

Effect of DMC on expression of cancer-relevant proteins

To gain knowledge on which cancer relevant signaling pathways that are affected by DMC, its impact on expression levels of the four cancer related proteins EGFR, P-gp, HIF-1α, and ERK was investigated in A549 cells under hypoxic and normoxic (normal) conditions. Western blot analysis revealed that for cells incubated under hypoxic conditions for 48 h the

level of the cancer relevant proteins EGFR, ERK and P-gp were increased compared to cells grown under normoxic conditions, confirming the effect of hypoxia on cancer relevant cell signaling. The addition 10 μg/mL of free DMC to the growth medium effectively suppressed the level of all investigated cancer related proteins relative to the control protein β-actin (**Fig. S2**). All of the proteins except ERK were suppressed to below the level in normoxic cells, but even the level of ERK was closer to that of normoxic than hypoxic cells.

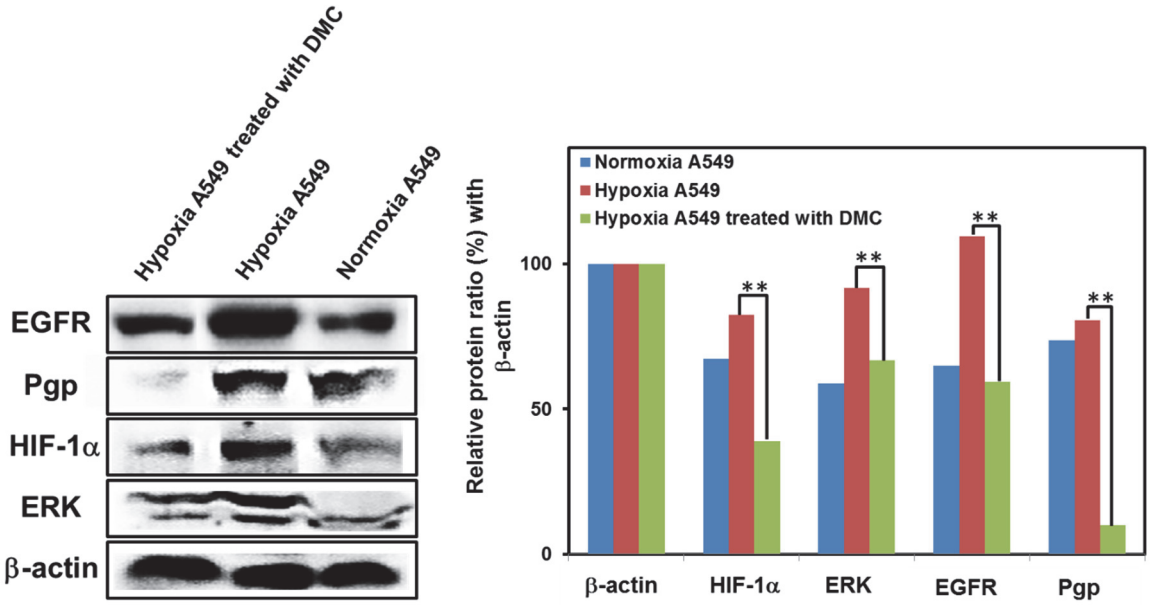

Fig. S2. *The effect of DMC on selected cancer related protein expression, analyzed by western blot (left). All western blotting bands were quantified using Image J and the relative quanteties are shown in the chart (right). Actin was used as loading control. Each data point is the average of three measurements. The double-star indicates significant difference ($p < 0.01$).*

Mice weight changing relating to section: 3.6. In vivo efficiency analysis

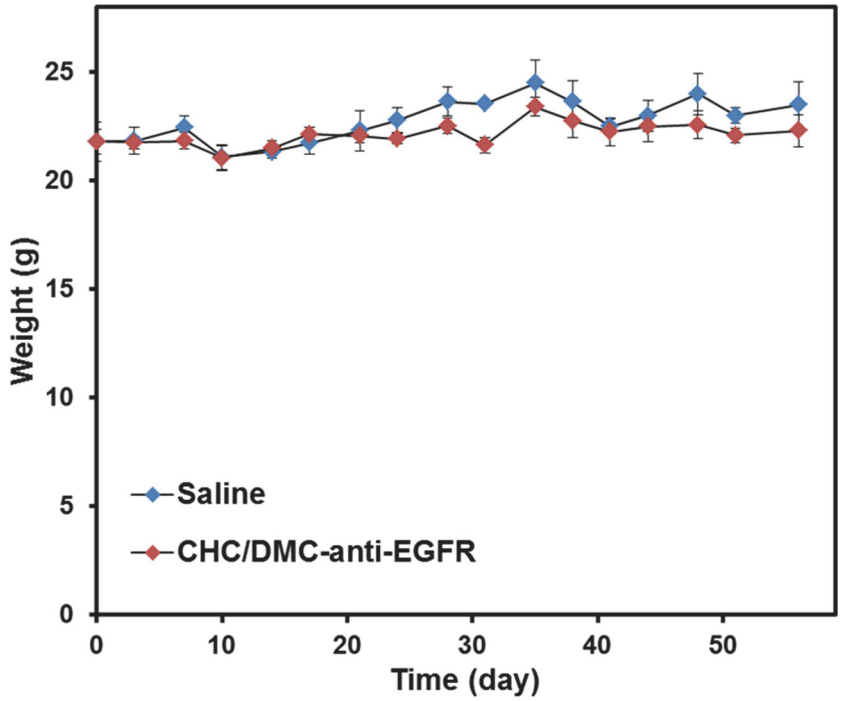

Fig. S3. *In vivo effect of CHC/DMC-anti-EGFR nanocarriers in an A549 tumor nude mice xenograft model. The weight change of the mice over time. Each data point is represented as mean ± SD (n = 5).*

1. Ritger, P. L.; Peppas, N. A. A simple equation for description of solute release I. Fickian and non-fickian release from non-swellable devices in the form of slabs, spheres, cylinders or discs. *J. Control. Release* **1987,** *5*, (1), 23-36.