Supporting Information

Nanostructured lipid carrier delivering sorafenib to enhance the immunotherapy induced by doxorubicin for effective esophagus cancer therapy

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Materials and methods

Chemicals, cell line and animal model

The chemicals and regents in our research were from Sigma (M.O. USA) unless otherwise stated. The doxorubicin (Dox) resistant mice lung carcinoma cell line (AKR/Dox) was purchased form ATCC (Virginia, USA) and cultured in standard DMEM (Gibco, California, USA) medium as reported previously. Male babl/c mice were obtained from Model Animal Research Center of Nanjing University (Nanjing, China) and all animal procedures were approved and supervised by the Animal Ethics Committee of Nanjing Medical University. The AKR/Dox tumor bearing mice was established according to previous report. The multicellular tumor spheroid (MCTS) was established according to previously reported protocol. Briefly, the mixed AKR/Dox and NIH3T3 (number ratio of 1:1) cells were seeded onto gel pre-coated 96 well plated and allowed to grow for 2-3 days into MCTS.

Preparation of nanoparticles

Glycerin monostearate (GM, 2 mg), phosphatidylcholine (PC, 3 mg), oleic acid (OA, 2 mg) and stearic acid-polyethylene glycol-folate (SA-PEG-FA, 3 mg) were added into 1 mL of ethanol and ultrasonicated (JY92-II, Ningbo Scientz Biotechnology Co., Ltd, China; 400 w, work 2 s and stand 3 s, 20 times) to obtain a transparent solution. To a preheated (70 °C) aqueous solution (10 mL), the mixture of lipid components was injected constantly (0.1 mL/s) via syringe under gentle agitation (500 rpm) and continue to agitate for another 10 min. The chemical drugs or fluorescence probe was loaded into NLCs by physical encapsulation. Drug or fluorescence probe was dissolved with lipids in the organic phase, and proceeded as described above. After drug loading, the solution was centrifuged at 20000 rpm for 10 min. The supernatant was collected and freeze-dried
to obtain powder. The powder was then dissolved in acetonitrile with appropriate heating, filtered through 0.45 μm membrane (Millipore, USA) and assessed by ultraviolet spectroscopy at 480 nm (Dox) and 265 nm (Sfn), respectively (UV5Nano, METTLER TOLEDO, Zurich, Switzerland) to determine the drug loading content.  

**Characterization**

The distribution of particle size was studied by a size analyzer (ZS90, Malvern, UK). The morphology was viewed by transmission electron microscope (TEM, JEM-2100Plus, JEOL, Japan). The stability of NLC/D-S was monitored by its size changes in PBS and mouse plasma for 48 h.

The release profile of Dox and Sfn from NLC/D-S, respectively, was investigated using previously reported protocol. The Dox concentration in the obtained buffer was determined by HPLC.

The hemolysis of nanoparticles was assessed according to previous report. In brief, 2% red blood cells suspension of New Zealand rabbit (Gibco) was incubated with different concentrations of NLC/D-S for 1 h at 37 °C and the supernatant collected after experiment (3000 rpm, 10 min) was assessed at 545nm by UV spectrophotometer.

In order to obtain the total protein, samples (cell or vehicle) lysed by RIPA buffer (Abcam, UK) as instructed by the manufacturer. The protein concentration in different samples was quantified and normalized. The samples were the loaded on SDS-PAGE gel for electrophoresis (120 V, 60 min). Afterwards, the protein bands were transferred onto PVDF membrane (90 V, 60 min), followed by staining with corresponding first antibodies (Abcam, UK). The IRDyeR680CW-labeled second antibody (Abcam, UK) was finally applied and the bands were visualized by Gel-Pro analyzer (Genegenuis, Syngene, UK).

**Cytotoxicity assay**

The cytotoxicity of various concentrations of drug unloaded nanoparticles (10-200 μg/mL) as well as NLC/D-S (Dox concentration, 2-50 μM the Sfn concentration: 2-50 μM, the molar ratio between Dox and Sfn was fixed at 1) on AKR/Dox cells for 48 h was studied using a standard MTT assay.

MCTS at the diameters between 300 and 400 μm were treated with fresh medium containing different formulation (Dox concentration 25 μM) for 5 days at 37 °C. The diameter changes of MCTS was recorded every day and plotted against time.

**Cellular uptake and in vivo distribution**

AKR/Dox cells were firstly seeded in 6-well plates with 70% confluence and then cultured free Dox or NLC/Dox for 2, 4 or 6 h. The cells were then subjected to observation under CLSM. In order to reveal the potential roles of NLC in the increase of cell uptake of DDS, AKR/Dox cells were incubated with additional FA for 2 h before the addition of free Dox or NLC/Dox. At pre-determined time intervals, cells were detached and positive cells were quantified using by flow cytometry (Quanteon, ACEA NovoCyte, Agilent, California, USA).

The ICG was loaded into the NLC along with drug loading and used to be the
probe to show the location of the DDS. Afterwards, AKR/Dox tumor bearing mice were administered with NLC/D-S intravenously. At different time interval after administration (4 and 8 h), the mice were sacrificed and the distribution of DDS was revealed by detecting the ICG signal in organ and tumor tissues using the *in vivo* imaging instrument (ZEWTON 7.0, Vilber, France).

**In vivo anticancer efficacy**

*In vivo* anticancer efficacy of NLC/D-S was explored using AKR/Dox tumor xenograft mice model. To be specific, mice were randomly divided into 5 groups (n = 6): 1) saline (as control); 2) NLC/Dox; 3) NLC/Sfn; 4) NLC/D-S. Protocols were adopted from previous report. Briefly, mice were intravenously injected with different formulations (Dox: 5 mg/kg; Sfn: 5 mg/kg) for every three days for 5 times until day 15 (primary tumor). The measurement of tumor volume was performed before each administration. Moreover, at the day 15, the same dose of tumor cells were planted on the other side of the mice and the tumor volume was recorded every three days for another 15 days (distant tumor). At the end of the tests, the tumor tissues were excised from sacrificed mice at the end of the test and subjected to Ki67 or TUNEL staining.

**In vivo immune activation of NLC/D-S**

The mice were administered with NLC/D-S as reported above. On day 0, 2 and 4 post PDT treatment, the blood of the subjects were collected and the Cytokine IL-6 related to the immune activation was determined using corresponding kits (Abcam, UK). The ICD of cells in the tumor tissues from different groups at day 15 was subjected to immunohistochemical staining.

**Sfn regulation on the TME, Tregs and effector T cells**

The mice were administered with NLC/Sfn at different dosage (2, 5 and 10 mg/kg) using the scheme in the *in vivo* anticancer experiment. At the end of the test, the tumor tissues were collected to prepare tumor-infiltrating lymphocytes (TIL) as reported previously. Afterwards, Purified CD4+ cells were then stained with CD4 and CD25-specific antibodies and the CD4+CD25+ were isolated by a FACSARia cell sorter (BD, San Jose, CA USA) and the population was calculated by cell counter (Countess II FL, Thermo. USA).

In order to further study the anti-proliferation and apoptosis effects of Sfn on Tregs, the sorted CD4+CD25+ Tregs were cultured in the presence of anti-CD3 (5 mg/mL), anti-CD28 (2 mg/mL) antibodies, IL-2 (100 U/mL) and the indicated concentrations of Sfn (2, 5 and 15 μM) in culture medium for 72 h. The cells were then harvested for [3H] thymidine incorporation assay (BD, San Jose, CA USA) staining as per instructions.

TIL were stained with anti-CD8 and anti-CD25 antibodies and sorted by flow cytometry. The expression of PD-1 ligand in CD8+ T cells was determined using specific primers. PD-1 ligand expression was normalized to the expression of the housekeeping gene HPRT. Primers include the following: PD-1 ligand, 5’- AATGCTGCCCCTTCAATACATCA-3’ (sense) and 5’-ACCCTCGGCGTCCGGAGA-3’ (antisense); HPRT, 5’-TCTCGAAGTGGTGGATAGGCCA-3’ (sense) and
5’CAACAGGACTCCTCGTATTTGCAG-3’ (antisense).

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