Electronic Supplementary Information for:

A shuttle/sink model composing of β-cyclodextrin and simvastatin-loaded discoidal reconstituted high-density lipoprotein for enhanced cholesterol efflux and drug uptake in macrophage/foam cells

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**Materials**

Soybean phospholipid was purchased from A.V.T. Pharmaceutical Co. Ltd. (Shanghai, China). Cholesterol and sodium cholate were obtained from Aladdin (Shanghai, China). β-cyclodextrin was obtained from Zhiyuan Biotechnology Co. Ltd (Shandong, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (USA). All reagents were of analytical grade except methanol and acetonitrile of chromatographic grade for HPLC analysis. Deionized or purified water was used in all experiments.

**S1. Determination of drug content**

The sample containing simvastatin was subjected to centrifugation by 12000 rpm for 10 min and the contents of the drug in the supernatant were measured by HPLC (Shimadzu, Japan). The HPLC system consisted of a Kromasil 100-5C_18 (150×4.6 mm) column, a UV-DAD detector (Shimadzu, Japan) operated at 238 nm and a pump (LC-20A). The mobile phase was methanol/water (78/22, v/v). The flow rate was 1.0 mL/min and the column temperature was set at 30°C.

**S2. Preparation of cholesterol donor**

Large unilamellar vesicle (LUV), serving as cholesterol donor to simulate cell membrane in acellular mimetic system, was prepared by thin film dispersion method as reported previously with minor modification. First, the mixture of soybean phospholipids (SPC) and cholesterol was dissolved in 10 mL of chloroform/methanol (3:1, v/v) and dried in an egg-plant flask under vacuum at 37°C for 1 h. After that, the obtained lipid film was hydrated by 10 mL Tris-HCl buffer (0.01 M, pH 8.0) containing sodium cholate. The weight ratio between SPC, cholesterol and sodium cholate was 100:30:13.5. After that, the suspension was ultrasonicated thoroughly for 10 min at 300 W in an ice bath by sonifier cell disrupter (JY92II, Ningbo, China). Finally, the cholesterol donor was obtained after dialyzing against Tris-HCl buffer for 6 h to remove the free cholesterol and sodium cholate, and then extruding the resultant suspension through 0.22 μm filter. LUV with an average diameter of 123.32 ± 3.72 nm was obtained and could keep stable for at least 3 days.

**S3. Determination of cholesterol and cholesteryl ester content**
The sample containing cholesterol was mixed with stigmasterol as the internal standard. Then cholesterol and stigmasterol were extracted with hexane and dried by nitrogen. After that, 1 mL of acetone was added to dissolve the residue and incubated with the mixture of chromic acid and sulfuric acid solution for 5 min for derivatization. Subsequently, the reaction was terminated by addition of 2 mL of hexane and 0.5 mL of water. The cholesterol derivative was extracted in hexane phase and dried by nitrogen. The final sample, dissolved in acetonitrile, was subjected to HPLC analyses. The HPLC system consisted of a Shim-pack VP-ODS column (5 μm, 150 mm×4.6 mm) column, a UV-DAD detector (Shimadzu, Japan) operated at 249 nm and a pump (LC-20A). The mobile phase was acetonitrile-water (90/10, v/v). The flow rate was 1.0 mL/min and the column temperature was set at 30°C.

For the measurement of cholesteryl ester, the sample was equally divided into two parts. One part was utilized directly to measure the amount of free cholesterol as described above. The other part was mixed with equal volume of 8.9 M of potassium hydroxide-ethanol (1:9, V/V) and incubated for 2 h at 50°C to hydrolyze cholesteryl esters into cholesterol. Then the amount of total cholesterol was determined as described above. The amount of cholesteryl ester was calculated by subtracting the amount of free cholesterol from the amount of total cholesterol.

### S4. Cell viability assay

Mouse macrophage cell line RAW 264.7 cells (kind gift from the Atherosclerosis Research Centre, Nanjing Medical University, Nanjing, PR China) were used in this experiment. The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin at 37±0.5°C and 5% CO₂. The macrophage/foam cells were obtained by incubating RAW 264.7 cells with 60 μg/mL of oxLDL for 24 h. The cytotoxicities of ST-d-rHDL and β-CD on RAW 264.7 cells and macrophage/foam cells was examined by MTT assay as reported previously. Briefly, RAW 264.7 cells or macrophage/foam cells were seeded in 96-well plates at a density of 5×10⁴ cells/well for 24 h. Then the medium was removed and replaced with 200 μL fresh serum-free DMEM containing β-CD (0.1~4 mM) and ST-d-rHDL (0.1~2
mg/mL of SPC) for 6 h at 37°C, respectively. After discarding the supernatant, 200 μL of MTT solution (500 μg/mL, in pH 7.4 PBS) was added into each well and incubated for 4 h at 37°C in dark. Subsequently, MTT solution were removed and washed with PBS for 3 times. Then, 150 μL of DMSO were added and shaken for 10 min. Optical density (OD) of each well were measured at 570 nm through a Microplate Reader. Untreated cells served as control group with 100% cell viability. All experiments were repeated 3 times. Cell viability (%) was calculated as the following equation.

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\text{Cell viability (\%) = } \frac{OD_S - OD_0}{OD_{\text{Control}} - OD_0}
\]

ODₘ stands for the OD of cells exposed to β-CD or ST-d-rHDL. ODₜₐₚₜ represents the OD of cells treated with supplement-free fresh culture medium and OD₀ represents the OD of wells without any treatment.

The results of MTT were showed in Fig S1, no obvious cytotoxicities were observed for β-CD at the concentration of 0.1~2 mM and ST-d-rHDL at the concentration of 0.1~1 mg/mL of SPC on RAW 264.7 cells. In term of macrophage/foam cells, no obvious cytotoxicities were observed for β-CD at the concentration of 0.1~3 mM and ST-d-rHDL at the concentration of 0.1~1.5 mg/mL of SPC. Considering that the macrophage/foam cells were in a pathological state characterized by the severe intracellular lipid deposition, the treatment of β-CD and ST-d-rHDL might benefit to the cell viability of macrophage/foam cells via reducing intracellular lipid deposition. As expected, cell viabilities of macrophage/foam cells after treated by of β-CD (0.5 and 1 mM) and ST-d-rHDL (0.2 and 0.5 mg/mL of SPC) were obvious higher than 100%. Therefore, 0.1, 0.5 and 2 mM of β-CD as well as ST-d-rHDL at the concentration of 0.5 mg/mL of SPC were used in other experiments.
In vitro cytotoxicities of (A) β-CD (0.1~4 mM) and (B) ST-d-rHDL (0.1~2 mg/mL of SPC) on RAW 264.7 cells and macrophage/foam cells (mean value ± SD, n=3).

**S5. Cholesterol removal ability normalized by the total cholesterol**

Firstly, RAW 264.7 cells were seeded in 24-well plates at a density of $1\times10^4$ cells per well overnight. Then the cell culture medium was renewed by fresh medium containing 60 μg/mL of oxLDL to induce the formation of macrophage/foam cells. After removing the medium and washing with PBS for 3 times, the cells were treated by following preparations: 0.5 mM of β-CD, ST-d-rHDL, ST-d-rHDL+0.1 mM of β-CD, ST-d-rHDL+0.5 mM of β-CD and ST-d-rHDL+2 mM of β-CD for 1, 2, 4, 6 h in the presence of LCAT, respectively. The amount of total cholesterol in the cell medium ($w$) and in the cell ($w_0$) was measured by HPLC. Cholesterol efflux (%) was calculated as following equation.

$$\text{Cholesterol efflux (\%)} = \frac{W}{W + W_0} \times 100\%$$  \hspace{1cm} (2)

As shown in Fig S2, combination of β-CD and ST-d-rHDL could significantly strengthen cholesterol efflux. The likely explanation was that β-CD could efficiently acquire cholesterol from cell membrane and transport to the carriers. The highest cholesterol efflux (%) was occurred in the group combining ST-d-rHDL with 0.5 mM of β-CD at 4 h, such results were consistent with the amount of cholesterol efflux corrected by the amount of cell protein.
Fig S2. Cholesterol efflux (%) from macrophage/foam cells normalized by the total cholesterol after incubating the cells with different preparations at the predetermined time points. *p < 0.05, **p < 0.01, ***p < 0.001 (mean value ± SD, n=3).

References

