Supporting Information

Nucleus-Targeted Protein Delivery via Lysine Dendron Conjugation

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Experimental Section

Materials and Reagents

All reagents and chemicals were purchased from commercial sources and used as received without further treatment. RNase A was purchased from Beyotime Biotechnology. Genistein and M-β-CD were purchased from Bide Pharmatech Ltd. Cy7-NHS-ester was purchased from Goyoo Bio Co., Ltd. (Nanjing, China). Fluorescein isothiocyanate (FITC) was bought from Beijing Ouhe Technology Co., Ltd.

Instruments

NMR data were recorded on Agilent 400MR-DD2, Dynamic Light Scattering (DLS) and Zeta potential was measured by NanoBrook Omni (Malvern, US). Fluorescence microscopy images were observed by Leica DM6. Tissue sections of H&E was imaged by Leica DMi8. Flow cytometry was performed on CytoFLEX. In vivo fluorescence was analyzed with an IVIS Lumina imaging system (PerkinElmer). Transmission electron microscope (TEM) was conducted by Hitachi H-7500 electron microscope. Images were acquired by fluorescent microscopy (Olympus IX 51, Japan).

Experimental Section

Synthesis of Compound 2

A mixture of tribromoneopentyl alcohol (1 g, 3.08 mmol) and sodium azide (0.84 g, 12.94 mmol) in N, N-dimethylformamide (DMF, 30 mL) was refluxed overnight. The crude product was concentrated, redissolved in acetone (30 mL), and filtered to remove salts. After evaporating acetone, the residue was dissolved in dichloromethane (DCM, 50 mL), washed with saturated NaCl solution (3 × 50 mL), dried over anhydrous Na_2SO_4, and concentrated to afford 580 mg of a colorless oily liquid (yield: 89%).

Synthesis of Compound 3

Compound 2 (580 mg, 2.75 mmol) and triethylamine (TEA, 573 μL, 4.13 mmol) were

dissolved in DCM (10 mL). p-Nitrophenyl chloroformate (831 mg, 4.13 mmol) was added dropwise at room temperature (RT), and the reaction was stirred for 4 h. The mixture was diluted with DCM (30 mL), washed with water (3 × 30 mL), dried, and concentrated. Purification by column chromatography (PE:EA = 10:1) yielded 859 mg of a white solid (yield: 83%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.31 (d, 2H), 7.40 (d, 2H), 4.19 (s, 2H), 3.46 (s, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 155.16, 151.93, 146.55, 125.38, 121.70, 67.35, 50.98, 43.46. LC-MS (m/z): $[M+Cl]^+$, calcd for 411.10, found: 411.07.

Synthesis of Compound 4

1,4,7-Triazacyclononane trihydrochloride (100 mg, 0.42 mmol) was dissolved in methanol (10 mL) with TEA (273 μL, 1.97 mmol). A solution of Compound 3 (268 mg, 0.71 mmol) in methanol (10 mL) was added dropwise at 0 °C. After stirring at RT for 2 h, the solvent was evaporated. The residue was dissolved in DCM (50 mL), washed with saturated $NaHCO_3$ (3 × 50 mL), dried, and purified by column chromatography (DCM:MeOH = 30:1) to give 160 mg of a colorless oil (yield: 63%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.06 (s, 4H), 3.59 (t, 4H), 3.38 (s, 12H), 3.33 (s, 2H), 3.28 (s, 2H), 2.92 (t, 2H), 2.88 (t, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 155.55, 64.03, 52.64, 52.47, 52.17, 51.56, 48.54, 48.30, 48.04, 43.69. LC-MS (m/z): $[M+H]^+$, calcd for 604.27, found: 604.28.

Synthesis of Compound 6N_3-S-S-PNZ

Compound 1 (131 mg, 0.27 mmol) was dissolved in DMF (5 mL) and cooled in an ice bath. A solution of Compound 4 (115 mg, 0.19 mmol) and TEA (40 μL, 0.29 mmol) in DMF (5 mL) was added dropwise. After 30 min at 0 °C, the reaction was warmed to RT for 2 h. The solvent was removed under reduced pressure, and the residue was dissolved in DCM (30 mL), washed with saturated $NaHCO_3$ (3 × 30 mL), dried, and purified by column chromatography (PE:EA = 2:1) to afford 148 mg of a colorless oil (yield: 82%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.29 (d, 2H), 7.40 (d, 2H), 4.54 (t, 2H), 4.39 (t, 2H), 4.05 (s, 4H), 3.50 (s, 12H), 3.38 (t, 12H), 3.03 (t, 2H), 2.98 (t, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 155.91, 155.74, 155.64, 155.32, 152.28, 145.45, 125.33,

121.78, 66.76, 64.64, 64.36, 63.04, 51.66, 51.49, 43.62, 37.66, 36.64. LC-MS (m/z): $[M+Na]^+$, calcd for 971.27, found: 971.26.

Synthesis of Compound DBCO-Lys

Compound 6 (100 mg, 0.33 mmol) was dissolved in DCM (10 mL) in a 25 mL round-bottom flask. Triethylamine (TEA, 69 μL, 0.50 mmol) and Boc-Lys(Boc)-OSu (175 mg, 0.40 mmol) were added, and the reaction was stirred at RT for 4 h. The mixture was diluted with DCM (30 mL), washed with saturated NaCl solution (3 × 30 mL), dried over anhydrous Na_2SO_4, and concentrated. Purification by column chromatography (PE:EA = 1:2) yielded 180 mg of a white powder, which was further dissolved in DCM (1 mL), and trifluoroacetic acid (TFA, 250 μL, 3.20 mmol) was added. The reaction was stirred at RT for 2 h, then concentrated under reduced pressure. The residue was repeatedly dissolved in methanol and evaporated (5×) to remove residual TFA. After further remove TFA with Et_3N, the product was precipitated with diethyl ether (3×) to afford 53 mg of a light brown solid (yield: 77%).^{1}H-NMR (400 MHz, D_2O): δ 7.46-7.30 (m, 8H), 5.11(d, 1H), 3.80-3.19 (m, 4H), 3.22-2.98 (d, 3H), 2.78-2.54 (m, 2H), 1.70-1.20 (m, 6H). ^{13}C-NMR (100 MHz, D_2O): δ169.52, 169.19, 155.75, 152.43, 151.32, 130.67, 128.96, 127.78, 126.55, 126.22, 124.41, 123.29, 120.71, 94.18, 75.92, 52.46, 38.72, 38.66, 38.02, 36.96, 30.58, 26.71, 21.59. LC-MS (m/z): $[M+Na]^+$, calcd for 435.53, found: 435.46.

Preparation of RNase A-LD Conjugate

Native RNase A (5 mg/mL) was reacted with $6N_3$-S-S-PNZ (10 equiv) in bicarbonate buffer (pH 8.0) at 4 °C for 12 h. After dialysis to remove excess $6N_3$-S-S-PNZ, the azide-modified RNase A was incubated with DBCO-Lys (20 equiv) in PBS (pH 7.4) at 25 °C for 6 h to get RNase A-LD. The conjugate was characterized by Zeta potential, TEM and DLS.

RNase A release study

For RNase A release experiment, RNase A-LD were treated with 10 mM DTT and incubated at 37 ºC. At predetermined time points (2, 4, 6, 12 and 24 h), the release of intact RNase A was measured by subjecting sample to 1.5 % agarose gel

electrophoresis.

Cell culture

Mouse breast cancer 4T1 cells were obtained from Shanghai Ek-Bioscience and cultured in complete RPMI-1640 medium (10% FBS, 1% penicillin/streptomycin) at 37 °C in the presence of 5% CO_2.

Cellular uptake

4T1 cells (density of 1×10^4 cells/well) were seeded in 96-well plates and incubated for 12 h at 37 °C under 5% CO_2. 100 μL fresh medium containing RNase A or RNase A-LD (RNase A was labeled with isothiocyanate (FITC)) was then added.After 2 h incubation, the cells were fixed with 4% paraformaldehyde, stained with DAPI and imaged with fluorescent microscopy (ix51, Japan).

Endocytosis mechanism

4T1 cells (density of 1×10^4 cells/well) were seeded in 96-well plates and incubated 12 h at 37 °C under 5% CO_2. The cells were incubated with different endocytosis inhibitor (10 mM NaN_3, 10 mM M-β-CD or 200 μM genistein) or placed at 4 °C for 1 h, and then RNase A-LD was added and incubated at 37 °C or 4 °C for another 2 h. After that cells were trypsinsized and collected for flow cytometry analysis.

Nucleus transport mechanism

To explore the nuclear transport mechanism of RNase A-LD, 4T1 cells were preincubated with ivermectin (25 μM) for 1 h and then RNase A-LD was added for further incubating another 2 h. After that, the cells were fixed with 4% paraformaldehyde, stained with DAPI and imaged with fluorescent microscopy.

In vitro cytotoxicity

4T1 cells (density of 1×10^4 cells/well) were seeded in 96-well plates and incubated for 12 h at 37 °C under 5% CO_2. Then the medium was replaced with fresh medium containing different concentration of free RNase A or RNase A-LD. After 24 h incubation, MTT assay was used to determine the cell viabilities.

Animal Model

All animal experiments were carried out in compliance with the requirements of the National Act on the Use of Experimental Animals (People's Republic of China) and were approved by the Experimental Animal Ethical Committee of Chongqing University Cancer Hospital. Female mice (6-8 weeks) were supplied by the Animal Center of Chongqing Medical University (Chongqing, China).

Biodistribution

4T1 tumor-bearing mice were injected intravenously with free RNase A or RNase A-LD (RNase A was labeled with Cy7). The mice were imaged at different time points postinjection by small animal imaging system (IVIS Lumina III, USA). 36 h after administration, the mice were sacrificed, major organs (heart, liver, spleen, lung and kidney) and tumors were imaged and analyzed by an IVIS lumina imaging system.

In vivo antitumor activity

For anti-tumor therapy studies, breast cancer 4T1 cells (2×10^6) were injected subcutaneously into female BALB/c-nude mice. When the tumor volume grew to approximately 70 mm^3, the mice were randomly divided into 3 groups (n=5): (1) PBS control group, (2) RNase A group and (3) RNase A-LD group. 150 μL of different samples with equal amount of RNase A (5 mg/kg) were intravenously injected into the mice every three days for a total of five times. The tumor volumes and weight were measured every two days for 16 days. The tumor volumes value was calculated as the following equation: tumor volumes = length × width2 × 0.5. At the end of experiment, the tumor was excised and weighed. For histological examination, tumor tissues and major organs (heart, liver, spleen, lung and kidney) were collected for hematoxylin and eosin (H&E) staining.

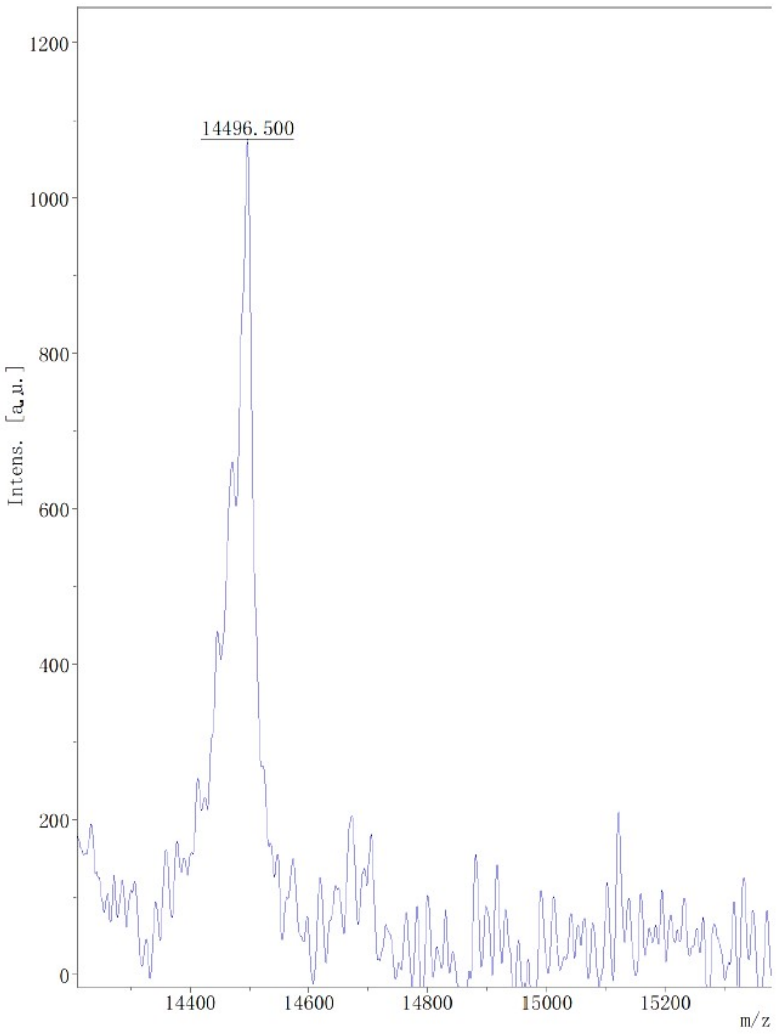

Figure S1. MALDI-TOF MS characterization of RNase A modification with $6N_3$-S-S-PNZ.

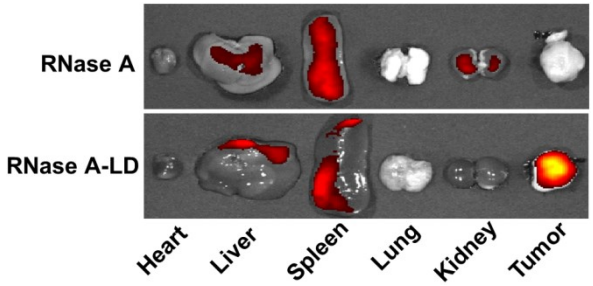

Figure S2. Ex vivo fluorescence images of tumors and major organs derived from the mice after 36 h injection.

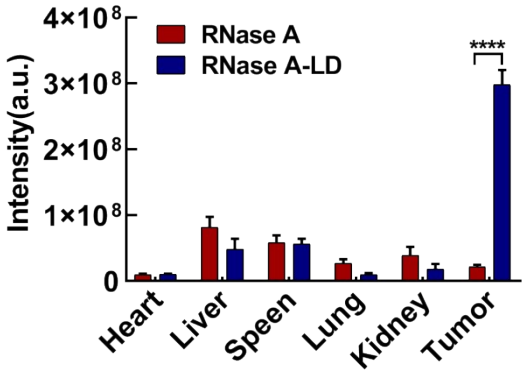

Figure S3. Quantification of the fluorescence intensities of the excised tumors and major organs of mice after 36 h injection.

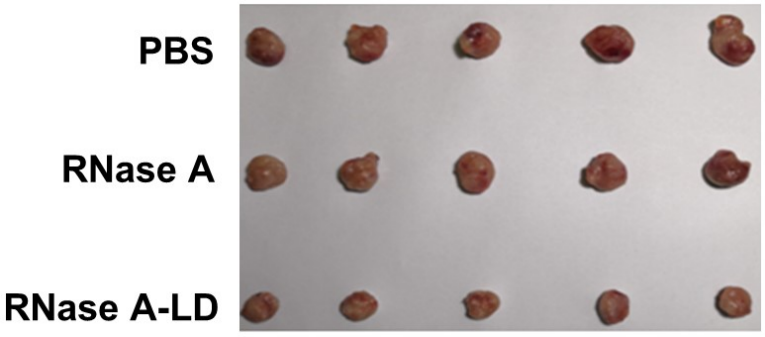

Figure S4. Photographs of tumors from mice after different treatments.

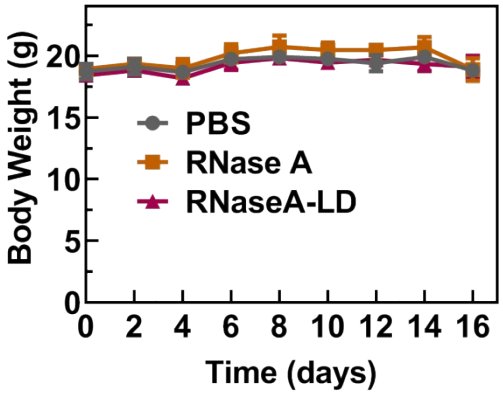

Figure S5. Variation in the body weight of mice during different treatments.

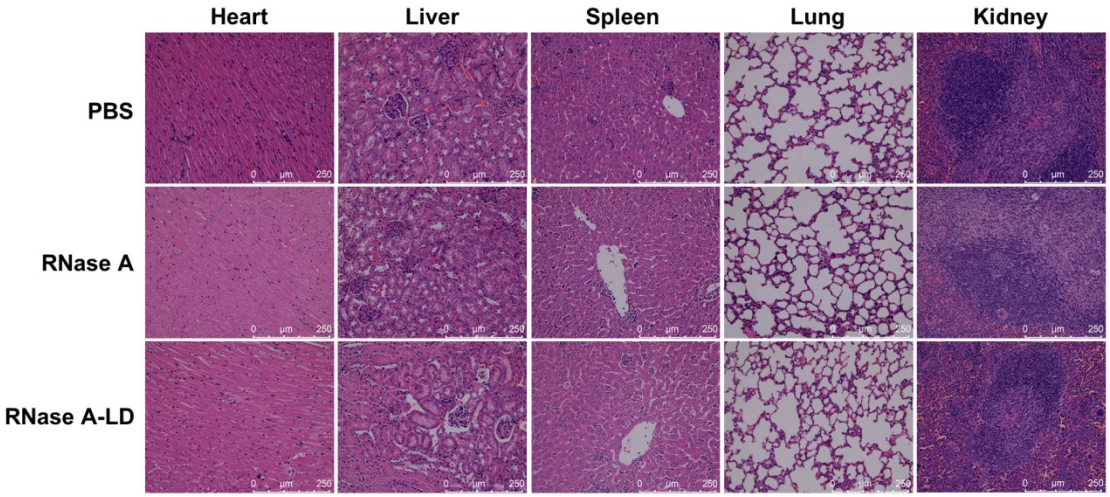

Figure S6. H&E staining of the main organs after different treatments. Scale bars: 250 μm.