Electronic Supplementary Information

Multifunctional dendrimers modified with alpha-tocopheryl succinate for targeted cancer therapy†

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Experimental

Materials

Ethylenediamine core amine-terminated G5 PAMAM dendrimer (G5.NH_2) with a polydispersity index less than 1.08 was purchased from Dendritech (Midland, MI). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), FA, and FI were from J&K Chemical Ltd (Shanghai, China). α-Tos was obtained from Hubei Hengshuo Chemical Co, Ltd. (Wuhan, China). 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich. All other solvents and reagents were of analytical grade and used as received. The water used in all the experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18 MΩ cm. Regenerated cellulose dialysis membranes with molecular weight cutoff (MWCO) of 10,000 were acquired from Fisher Scientific (Hudson, NH). KB cells (a human epithelial carcinoma cell line) were from the Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Unless otherwise specified, all cell culture media and reagents were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China).

Synthesis of G5.$NHAc_n$-α-Tos-FI-FA conjugate

The synthesis of G5.$NHAc_n$-α-Tos-FI-FA conjugate is schematically illustrated in Scheme 1. First,

G5.NH_2-α-Tos conjugate was synthesized through formation of amide bonds between the primary amines of G5 dendrimer and the carboxyl groups of α-Tos via an EDC coupling reaction. In brief, α-Tos (11.1 mg, 2.1×10^{-5} mol, 3 mL in dimethyl sulfoxide (DMSO)) was mixed with a DMSO solution of EDC (20.2 mg, 1.1×10^{-4} mol, 3 mL) under magnetic stirring at room temperature for 3 h. The resulting solution of activated α-Tos was dropwise added into a DMSO solution of G5.NH_2 (55 mg, 2.1×10^{-6} mol, 5 mL) under vigorous magnetic stirring at room temperature for 3 days. After that, the reaction mixture was dialyzed against a gradient ethanol solution of 40%, 30%, 20%, 10% (each for one time, 1 L, and 24 h) and water for 48 h (2 L, 2 times) via a dialysis membrane with MWCO of 10,000, followed by lyophilization to get G5.NH_2-α-Tos conjugate as a white powder. The product was stored at -20 °C before use.

To synthesize the G5.$NHAc_n$-α-Tos-FI-FA conjugate, the above G5.NH_2-α-Tos conjugate was sequentially modified by acetic anhydride, FI, and FA according to the procedure described in our previous reports.[1-3] Briefly, the G5.NH_2-α-Tos conjugate (50.0 mg, 1.7×10^{-6} mol) dissolved in DMSO (5 mL) was mixed with triethylamine (34.1 μL, 2.4×10^{-4} mol) under vigorous magnetic stirring for 30 min. Then, acetic anhydride with 95 molar equivalents to G5 dendrimer (15.5 μL, 1.6×10^{-4} mol) was dissolved in DMSO (5 mL) and dropwise added into the dendrimer/triethylamine mixture solution under vigorous magnetic stirring at room temperature. The reaction was continued for 1 day. Then the reaction mixture was dialyzed against phosphate-buffered saline (PBS, 3 times, 2 L) and water (3 times, 2 L) for 3 days through a dialysis membrane with MWCO of 10,000, followed by lyophilization to obtain the partially acetylated G5.$NHAc_n$-α-Tos conjugate. After that, the obtained G5.$NHAc_n$-α-Tos conjugate (40.0 mg, 1.2×10^{-6} mol) was dissolved in DMSO (5 mL) and reacted with FI (3.8 mg, 7.2×10^{-6} mol, dissolved in 3 mL DMSO) under magnetic stirring at room temperature for 24 h to get the raw product of G5.$NHAc_n$-α-Tos-FI conjugate.

Finally, EDC-activated FA produced by mixing the FA (4.2 mg, 9.6 × 10^{-6} mol, 3 mL in DMSO) and EDC (18.4 mg, 9.6 × 10^{-5} mol, 3 mL in DMSO) at room temperature for 4 h was dropwise added to the raw product of G5.NHAc$_n$-α-Tos-FI conjugate under magnetic stirring at room temperature. The reaction was stopped after 3 days. The final reaction mixture was dialyzed against PBS (3 times, 2 L) and water (3 times, 2L) for 3 days, followed by lyophilization to obtain the G5.NHAc$_n$-α-Tos-FI-FA product as an orange solid.

Characterization techniques

^{1}H NMR spectra were recorded on a Bruker AV400 nuclear magnetic resonance spectrometer. Samples were dissolved in D_2O before measurement. UV-Vis spectroscopy was performed using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, Boston, MA). All dendrimer samples were dissolved in water at a concentration of 0.5 mg/mL before measurements. For comparison, free α-Tos was dissolved in ethanol before the measurement. Zeta potential measurements were carried out using a Zetasizer Nano ZS system (Malvern, Worcestershire, UK) equipped with a standard 633 nm laser. Dendrimer samples were measured under different pH conditions (pH 5.0, 7.0, and 10.0, respectively) with a concentration of 1 mg/mL.

Cell biological evaluation

KB cells were continuously grown in two 50-mL culture flasks, one in FA-free medium and the other in regular RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin, and 2.5 μM of FA. The cells cultured in FA-free medium for 24 h expressed high level of FAR (denoted as KB-HFAR), whereas the cells grown in FA-containing medium expressed low level FAR (denoted as KB-LFAR). Note that the term of "KB cells" denotes KB-HFAR cells, in the context to differentiate the two types of KB cells, the terms of KB-HFAR and KB-LFAR are then used.

To test if the G5.NHAc$_n$-α-Tos-FI-FA conjugate was therapeutically effective, the KB cells were plated into a 96-well plate (1×10^4 cells per well) in a complete medium at 37 °C and 5% CO_2 one day before the experiments. The next day, the medium was respectively replaced with 200 μL of FA-free medium containing PBS (10 μL), ethanol (1 μL), G5.NHAc-FI-FA in PBS solution (10 μL), free α-Tos in ethanol solution (1 μL), and G5.NHAc$_n$-α-Tos-FI or G5.NHAc$_n$-α-Tos-FI-FA conjugate in PBS (10 μL). The final α-Tos concentration for both free drug and dendrimer conjugate was controlled at 12.5, 25, 50, 100, 200 μM, respectively. MTT assay was carried out after the cells were incubated for 48 h at 37 °C. After treatment with different samples for 48 h, the morphology of cells was observed using a Leica DM IL LED inverted phase contrast microscope with a magnification of 200× for each sample.

The targeting of the dendrimer conjugate to cancer cells was first confirmed by laser scanning confocal microscopy. Cover slips with a diameter of 14 mm were pretreated with 5% HCl, 30% HNO_3, and 75% alcohol and then fixed in 12-well tissue culture plate. 4×10^4 KB cells were seeded into each well and cultured for about 48 h to allow the KB cells to attach onto the cover slips. Before imaging, G5.NHAc$_n$-α-Tos-FI-FA and G5.NHAc$_n$-α-Tos-FI conjugate with the same α-Tos concentration (0.4 μM) was separately added to both KB-HFAR and KB-LFAR cells. After 3 h incubation, the medium in the wells containing dendrimer-α-Tos conjugate was entirely removed and replaced with the same volume of fresh medium without the dendrimer conjugates. KB-HFAR cells treated with PBS were used as control. Then the KB cells were rinsed with PBS for 3 times, fixed with glutaraldehyde (2.5%) for 15 min at 4 °C, and counterstained with Hoechst 33342 (1 μg/mL) for 15 min at 37 °C using a standard procedure. The FI fluorescence was excited with a 488 nm argon blue laser, and the emission was collected through a 505-525 nm barrier filter. Finally, samples were imaged using a 63 × oil-immersion objective lens.

The targeted uptake of G5.NHAc$_n$-α-Tos-FI-FA conjugate by cancer cells overexpressing FAR was further assessed by flow cytometry. Approximately 1×10^5 cells (both KB-HFAR and KB-LFAR) per well were separately seeded in 12-well plates the day before the experiments to bring the cells to confluence. The medium was then replaced with fresh medium containing G5.NHAc$_n$-α-Tos-FI-FA and G5.NHAc$_n$-α-Tos-FI conjugates with the same α-Tos concentration (0.4 μM). After 1 h incubation, the medium in wells was totally taken out, and the cells were rinsed with PBS for 3 times, trypsinized, resuspended in PBS, and analyzed using a Becton Dickinson FACSCalibur flow cytometer equipped with an argon laser (488 nm). The FL2-fluorescence of 10000 cells was determined, and the mean fluorescence of the gated viable cells was quantified.

In order to quantify the viability of the cells, an MTT assay was performed. Metabolically active cells were detected by adding MTT to each well after 48 h incubation with free α-Tos or dendrimer-α-Tos conjugate to determine the therapeutic effect of the dendrimer-α-Tos conjugate. To evaluate the targeted cancer cell inhibition effect, both KB-HFAR and KB-LFAR cells were plated into a 96-well plate (1×10^4 cells per well) in a complete medium at 37 °C and 5% CO_2 one day before the experiments. The next day, the medium was replaced with 200 μL of FA-free medium containing PBS (10 μL), free α-Tos (1 μL in ethanol, [α-Tos] = 50 μM), G5.NHAc$_n$-α-Tos-FI (10 μL in PBS, [α-Tos] = 50 μM), or G5.NHAc$_n$-α-Tos-FI-FA (10 μL in PBS, [α-Tos] = 50 μM) conjugate, respectively. After 3 h incubation, the medium was replaced with fresh drug-free medium and the cells were cultured for additional 48 h before MTT assay. For MTT assay, unattached cells were washed out with PBS solution and MTT solution (10 μL) was added to each well. After incubation at 37 °C for 4 h, 100 μL of DMSO was added to dissolve the purple MTT formazan crystal. Then, the plates were read at 570 nm using a Microplate Reader (MK3, Thermo). Mean and standard deviation of 6 parallel wells for each sample were reported.

Statistical analysis

One way ANOVA statistical analysis was performed to evaluate the experimental data. 0.05 was considered as statistical significance level, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

References

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2. X. Shi, I. Bányai, K. Rodriguez, M. T. Islam, W. Lesniak, P. Balogh, L. P. Balogh and J. R. Baker, Jr., *Electrophoresis*, 2006, **27**, 1758-1767.
3. X. Shi, W. Lesniak, M. T. Islam, M. C. MuÑiz, L. P. Balogh and J. R. Baker, Jr., *Colloid Surf. A-Physicochem. Eng. Asp.*, 2006, **272**, 139-150.

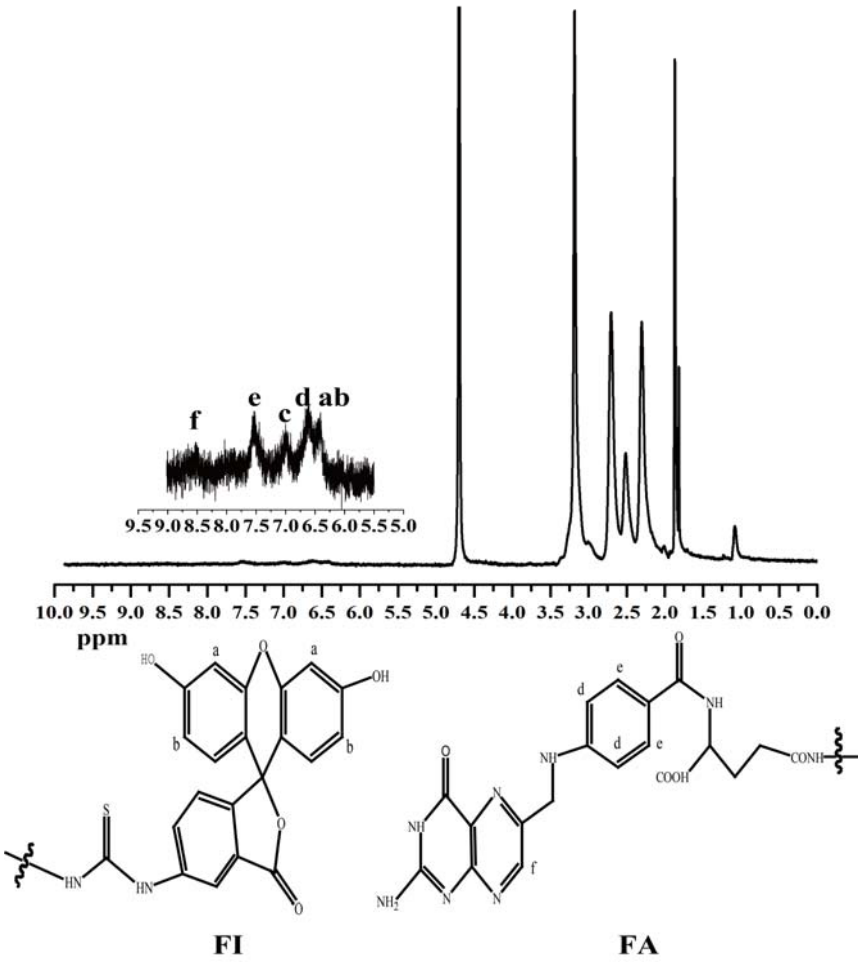

Figure S1. ^{1}H NMR spectrum of G5.NHAc$_n$-FI-FA dendrimer.

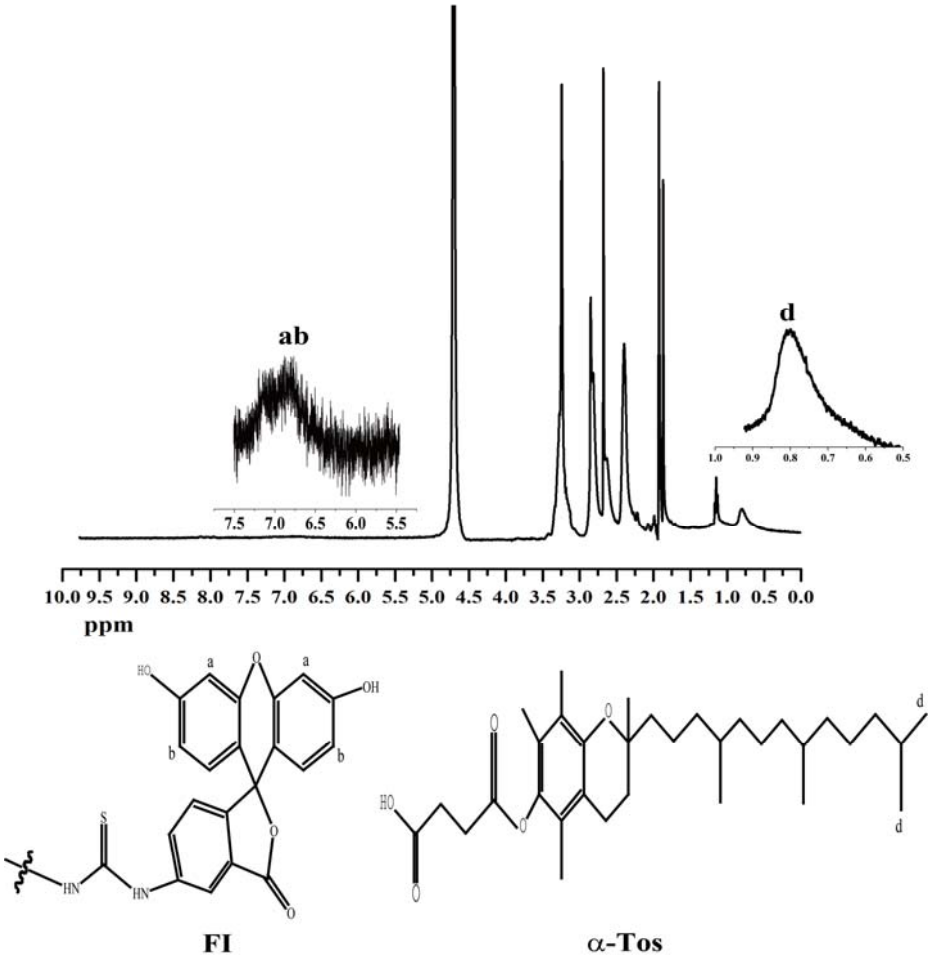

Figure S2. ^{1}H NMR spectrum of G5.NHAc$_n$-α-Tos-FI conjugate without FA.

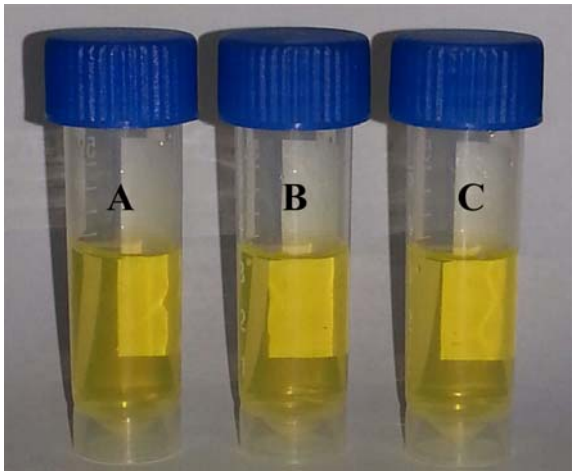

Figure S3. The photograph of G5.NHAc$_n$-α-Tos-FI-FA conjugate dispersed in water with a pH value of (A) 5.0, (B) 7.0, and (C) 10.0, respectively.

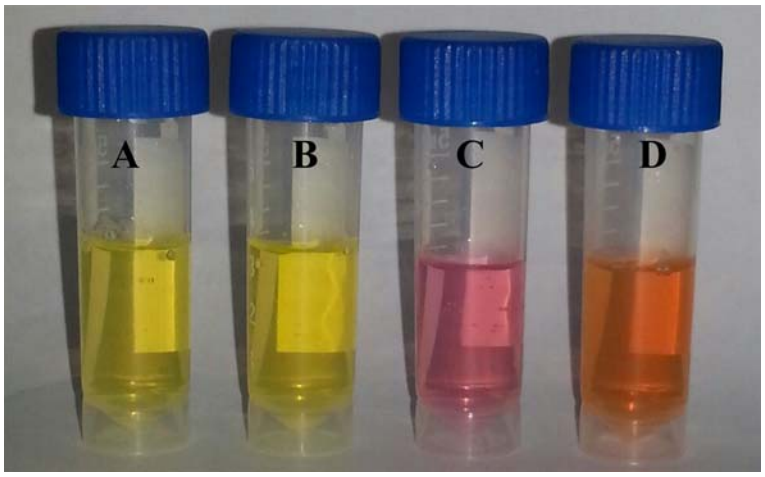

Figure S4. G5.NHAc$_n$-α-Tos-FI-FA conjugate dispersed in (A) water, (B) PBS, and (D) cell culture medium. (C) shows the blank cell culture medium.

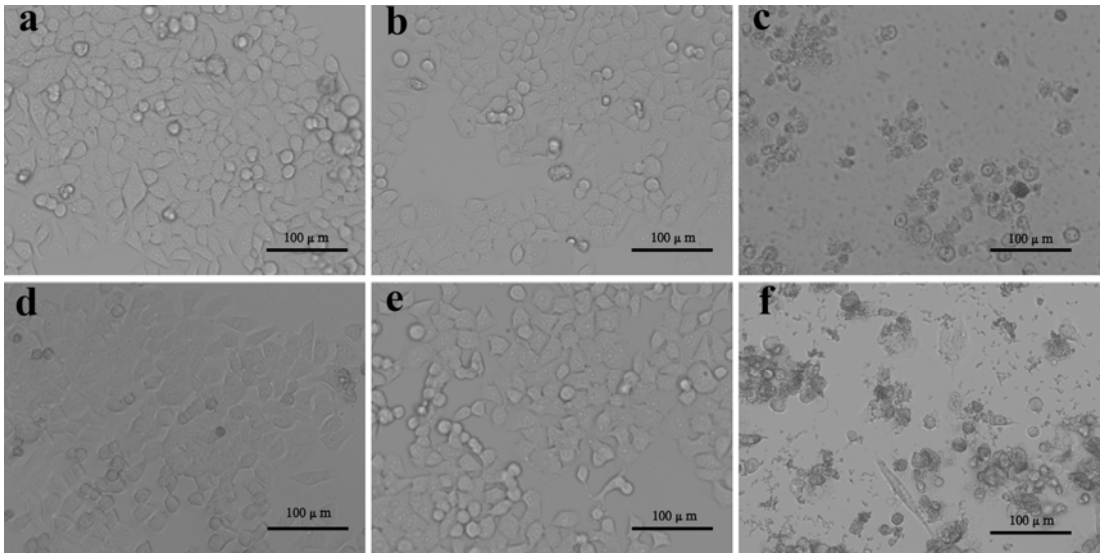

Figure S5. Phase contrast microscopic images of KB cells without treatment (a) and KB cells treated with (b) 1 μL ethanol, (c) free α-Tos in 1 μL ethanol (50 μM), (d) 10 μL PBS, (e) G5.NHAc$_n$ -FI-FA dendrimers in 10 μL PBS, and (f) G5.NHAc$_n$-α-Tos-FI-FA conjugate with α-Tos concentration of 50 μM in 10 μL PBS, respectively.

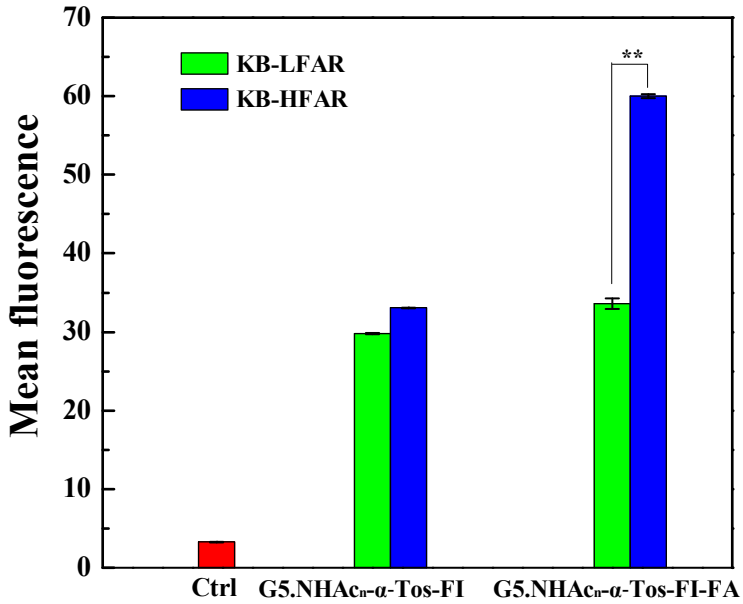

Figure 6. Flow cytometry analysis of the mean fluorescence of KB-HFAR and KB-LFAR cells treated with G5.NHAcn-α-Tos-FI and G5.NHAcn-α-Tos-FI-FA conjugates with α-Tos concentration of 0.4 μM for 1 h. KB-HFAR cells treated with PBS were used as control.