Supporting Information

Development of a novel albumin-based and maleimidopropionic acid conjugated peptide with prolonged half-life and increased in vivo anti-tumor efficacy

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Materials and methods:

Cell ELISA

HUVECs were seeded in 96-well plate (2×10^4 cells/well) and incubated overnight. Then cells were fixed with 100 µl fixation buffer (25% glutaraldehyde, 1:200 diluted with PBS) at RT for 20 min. After blocking with 5% nonfat milk in PBS for 2 h at 37°C, cells were washed 3 times with PBS, and then different concentration of peptides were added in each well and incubated for 1 h at 37°C. Cells were washed 3 times and incubated with 9G10-HRP. After reaction with OPD and terminated with 12.5 % H2SO4, the absorbance in each well was measured at 490 nm by a microplate reader.

CAM assay.

Upon reception, fertilized eggs of white Leghorn chickens (Meri Avignon Laboratory Animal Technology, Beijing, China) were incubated at 37°C in a 60% humidified atmosphere to trigger embryo development. After incubation for 72 h, eggs were sterilized and opened with an electric drill. The whole egg contents were removed into Petri dishes, and incubated at 37.5°C. A filter disk of 6-mm diameter containing 10 µl PBS or different peptides was placed onto the CAM of the embryos. At least 5 embryos were used for each group. After 48 h of incubation, vessel formation was observed under a stereomicroscope around the filter papers. The formation of vessels were quantified by comparing the vascular area to the CAM area (corresponding to the area of the 0.5-cm diameter filter paper) using Image-Pro Plus 6.0 software.

MTT assay

MTT assay was performed to determine cell viability. Briefly, HUVEC cells were seeded into 96-well plates at a density of 1500 cells/well. The cells were cultured overnight for adherence to the surface of the plate and treated with vehicle or indicated drugs and incubated at 37°C in 5% CO2 for 72 h. Then MTT assay was performed according to the manufacturer's instructions. The absorbance was read at 490 nm with a microplate reader (Bio-Rad, Richmond, CA). The mean values were calculated based on the data of six replicates.

Biodistribution and micro-PET imaging studies

The syntheses of DOTA-F56 and DOTA-F56-CM were accomplished using a solid phase synthesis strategy on a CS Bio CS336 instrument (Menlo Park, CA, USA). 64Cu (7.4 MBq /µl) was supplied by the Department of Nuclear Medicine, Peking University Cancer Hospital & Institute in Beijing [Ref 1]. The radiolabeling of DOTA-F56 by 64Cu was performed as described before [11]. Radio-TLC was performed with silica-impregnated glass fiber sheets (TLC-SG) (Agilent, USA) cut to 2.0 x10 cm in size and 0.01M EDTA contained 0.9% saline was
used for elution. Radioactivity was determined with radioactive thin-layer chromatography scanner (Bioscan, IAR-2000, Washington DC, USA). The Agilent HPLC (Santa Clara, CA, USA) system was equipped with a dual-wavelength absorbance detector, an in-line radioactivity detector, and a size exclusion Agilent Bio-SEC 3 column (4.6 mm × 300 mm, 150 Å). 0.01 M pH 7.4 sodium phosphate buffer (PBS) was used as a mobile phase. The HPLC conditions: solvent A, 0.01% TFA in QC water and solvent B, 0.01% TFA in CH₃CN. The gradient HPLC program: 0-10 min solvent B from 20% to 65%. The flow rate was 1.0 ml/min. The radioactivity was measured using an automated gamma scintillation counter (Perkin Elmer 1470-002, PerkinElmer, Waltham, MA, USA).

For the biodistribution studies, mice bearing BGC-823 or HT-29 tumors in each group were injected intravenously with ¹⁶⁴Cu-DOTA-F56 (0.74 MBq, 0.02 nmol, 0.1 ml) or ¹⁶⁴Cu-DOTA-F56-CM (0.74 MBq, 0.02 nmol, 0.1 ml) and sacrificed by decapitation at 4 h after injection. Blood, tumor and other organ samples were removed, weighed and counted in an automatic gamma counter. The results were expressed in terms of the percentage of injected activity per gram (%IA/g) of blood or organs. Small animal PECT/CT imaging studies were carried out using a micro-PET scanner (Siemens Medical Solutions, Malvern, PA, USA). Nude mice bearing BGC-823 or HT-29 xenografts were injected with ¹⁶⁴Cu-DOTA-F56 (37 MBq, 1 nmol, 0.1 ml) or ¹⁶⁴Cu-DOTA-F56-CM (37 MBq, 1 nmol, 0.1 ml) via tail vein. At 4 h after injection, the mice were anesthetized with 1.5% isoflurane and placed in the prone position, near the center of the field of view of micro-PET. The 10-min static scans were obtained, and the images were reconstructed by a two-dimensional ordered subsets expectation maximum (OSEM) algorithm. No background correction was performed.

**In vitro stability test**

The stabilities of ¹⁶⁴Cu-DOTA-F56 and ¹⁶⁴Cu-DOTA-F56-CM were evaluated in 0.01 M PBS (pH 7.4), 0.1 M NaAc (pH 5.5), or 5% HSA for a total of 24 h post-incubation. Specifically, each reaction mixture was incubated at 37°C with shaking in a thermo mixer and the chemical composition was determined by radio-HPLC at different time points during the 24 h incubation, with three repeats for each time point.

**In vitro cell uptake assay**

Cells were seeded at 2.0 × 10⁵ per well onto 24-well plates and allowed to attach on the surface overnight. Cells were washed twice with serum-free medium and incubated with radiotracer (1 µCi per well, 37 kBq) in 400 µl of serum-free medium at 37°C. After various time points (10 min, 30 min, 60 min and 120 min), cells were washed three times with cold PBS and lysed with 200 µl of 0.1 M NaOH. Radioactivity of the cells was counted using a
PerkinElmer 1470 automatic γ-counter. Cell uptake data were expressed as the percentage of the applied radioactivity per total radioactive.

Reference

Fig. S1. F56-CM conjugated with HSA in vitro. (A) Representative MALDI chromatograms of F56-CM adducts with different amounts of HSA. The ratio of F56-CM to HSA ranging from 3:1 to 1:5 (mol/mol). (B) Western blot analysis of F56 or F56-CM incubated with HSA in vitro.
Fig. S2. F56-CM conjugated with MSA and inhibited HUVEC tube formation and migration. (A) MALDI mass spectra of MSA or peptides adducts with MSA after 3 h incubation. The measured molecular weight was consistent with the expected molecular weight and marked next to the peak. (B) Western blot analysis of F56 or F56-CM adducted with MSA. (C) Tube formation assay. HUVEC cells were pre-incubated with peptides for 30 min before exposure to VEGF (10 ng/ml) and then seeded on the matrigel. After 6 h of incubation, formation of tube-like structures were photographed from randomly chosen fields (left) and the total pipe length of the tube-like structure were quantified (right). (D) Migration assay. HUVEC cells were starved for 24 h and then seeded into the transwell insert in the presence of indicated doses of peptides for 8 h. Cells reaching the bottom side of the transwell membrane were stained and representative images were displayed (left, Scale bar: 200 μm) and the migration cell number of HUVEC were quantified (right).

Mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. S3. In vitro stabilities and cell uptake analysis of $^{64}$Cu-DOTA-F56 and $^{64}$Cu-DOTA-F56-CM. (A) $^{64}$Cu-DOTA-F56 or $^{64}$Cu-DOTA-F56-CM was mixed with 0.01 M PBS (pH 7.4), 0.1 M NaAc (pH 5.5), or 5% HSA, respectively. After incubation at 37°C with shaking in a thermo mixer, the radiochemical purity was measured by Radio-HPLC. (B) Cell uptake analysis of $^{64}$Cu-DOTA-F56 and $^{64}$Cu-DOTA-F56-CM tracer in HUVEC, BCG-823, HT-29 cell lines at 10 min, 30 min, 1 h and 2 h. The data were expressed as the percentage of the applied radioactivity per total radioactive.

Mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.