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An innovative pre-targeting strategy for tumor cell specific imaging and therapy†

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Materials

Transferrin-biotin, fluorescein isothiocyanate-avidin (FITC-avidin), avidin and biotin were purchased from Sigma-Aldrich. 12-aminododecanoic acid (NH_2-$C_{11}H_{22}$-COOH, ADDA), trifluoroacetic acid (TFA) and other reagents were provided by Shanghai Chemical Co. (China). Fluorenylmethoxycarbonyl (Fmoc) protected D-amino acids (D-Fmoc-Lys-OH, D-Fmoc-Leu-OH, D-Fmoc-Val-OH), L-amino acids (Fmoc-Gly-OH, Fmoc-Lys(Mtt)-OH), Rink Amide-AM resin (100-200 mesh, loading: 0.59 mmol·g^{-1}, 1% DVB), O-benzotriazole-N,N,N',N'- tetramethyluronium-hexafluoro-phosphate (HBTU), N, N-diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), and piperidine were all obtained from GL Biochem Ltd. (Shanghai, China). Rhodamine B (RhB) was purchased from Aladdin. Dulbecco's phosphate buffered saline (PBS), Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), the mitochondria fluorescence probe (Mito Tracker Green FM), trypsin, antibiotic penicillin-streptomycin, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zoliumbromide (MTT) were purchased from Invitrogen Corp. YOYO-1 iodide was received from Molecular Probes (Eugene, OR) and prepared to 1 mM solution with DMSO. Fluorescent dye of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide) was also purchased from Sigma-Aldrich. All other chemical agents were obtained from Sinopharm Chemical Reagent Co., Ltd. and used as received.

1. Synthesis of Fmoc-$C_{11}H_{22}$-COOH (ADDA)

Fmoc-$C_{11}H_{22}$-COOH was obtained by protecting the amino group of NH_2-$C_{11}H_{22}$-COOH with Fmoc group according to the literature.[S1] The purification of the product was carried out via the recrystallization from acetonitrile. ^{1}H NMR spectrum of ADDA was performed on a Mercury VX-300 spectrometer at 300 MHz (Varian,

USA). DMSO-d6 was used as the solvent. ^{1}H NMR (300 MHz, DMSO-d6 (ppm)): 7.25-7.90 (m, 12 H), 4.17-4.37 (m, 3H), 2.94 (t, 2H), 2.13 (t, 2H), 1.23-1.47 (m, 18H). The ^{1}H NMR spectrum is shown in Fig. S1.

2. Peptide synthesis and characterization

Peptides (P1: $_D$(KLAKLAK)$_2$, P2: Fmoc-(ADDA)-Gly-$_D$(KLAKLAK)$_2$-Ala-Lys, P3: Fmoc-(ADDA)$_2$-Gly-$_D$(KLAKLAK)$_2$-Lys(biotin), P4: RhB-(ADDA)$_2$-Gly-$_D$(KLAKLAK)$_2$-Lys(biotin)) were synthesized by the mellow manual Fmoc solid phase peptide synthesis (SPPS) protocol. P1 and P2 were synthesized according to the literature.[26] To prepare the biotinylated pro-apoptotic peptides (P3, P4), Fmoc-Lys(Mtt)-OH was first manually attached to the Rink Amide-AM resin. Selective removal of the Mtt protecting group was accomplished by agitating the resin in a 1:99 (v/v) TFA/DCM solution for 5 min, and repeated the cleavage operation until the filtrate turned to be colorless. Biotin was fused to the resulting free ε-amine using the HBTU chemistry (2 equiv. of biotin, 2.4 equiv. of HBTU and 6 equiv. of DIEA in DMF/DMSO). The following coupling of the peptides was then completed according to the traditional peptide synthesis method. The molecular weights of the peptide and their amphiphiles were analyzed by electrode spray ionization mass spectrometry (ESI-MS) or matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). P1: ESI-MS: calculated 1522.08, found 762.5 and 1523.8, which correspond to the pattern of $[M+H]^+$ and $[M+H]^{2+}$, respectively; P2: MALDI-TOF-MS: calculated 2198.86, found 2198.16; P3: MALDI-TOF-MS: calculated 2551.40, found 2550.34; P4: MALDI-TOF-MS: calculated 2787.82, found 2752.40, which corresponds to the pattern of $[M-Cl]^-$. P1: ^{1}H NMR (300 MHz, D_2O (ppm)): 4.24-4.37 (m, -NH-C***H***($CH_2CH(CH_3)_2$)-CO-; -NH-C***H***($(CH_2)_4$-NH_2)-CO-; -NH-C***H***(CH_3)-CO-), 2.95-2.97 (m, -NH-CH($(CH_2)_3$-C***H***$_2$-NH_2)-CO-), 3.48-3.62 (m, NH_2-C***H***($(CH_2)_4$-NH_2)-CO-

), 1.34-1.87 (m, -NH-CH((C***H***$_2$)$_3$-CH$_2$-NH$_2$)-CO-; -NH-CH(C***H***$_2$C***H***(CH$_3$)$_2$)-CO-; -NH-CH(C***H***$_3$)-CO-), 0.84-0.91 (m, -NH-CH(CH$_2$CH(C***H***$_3$)$_2$)-CO-). P2: ^{1}H NMR (300 MHz, DMSO (ppm)): 8.16-7.87 (m, -CO-N***H***-), 7.06-7.67 (m, ***Fmoc***), 3.93-4.25 (m, -NH-C***H***(CH$_2$CH(CH$_3$)$_2$)-CO-; -NH-C***H***((CH$_2$)$_4$-NH$_2$)-CO-; -NH-C***H***(CH$_3$)-CO-), 2.95-2.97 (m, -NH-CH((CH$_2$)$_3$-C***H***$_2$-NH$_2$)-CO-), 3.48-3.62 (m, NH$_2$-C***H***((CH$_2$)$_4$-NH$_2$)-CO-), 1.20-1.66 (m, -NH-CH((C***H***$_2$)$_3$-CH$_2$-NH$_2$)-CO-; -NH-CH(C***H***$_2$C***H***(CH$_3$)$_2$)-CO-; -NH-CH(C***H***$_3$)-CO-), 0.81 (m, -NH-CH(CH$_2$CH(C***H***$_3$)$_2$)-CO-).

3. Characterization of peptide and their amphiphiles

Circular dichroism (CD, Jasco J-810 spectropolarimeter, Japan) and Fourier transform infrared spectroscopy (FT-IR, Perkin-Elmer Spectrum One, USA) were employed to investigate the secondary structure of P1-4. Sodium dodecyl sulfate solution (SDS, 25 mM) was used to prepare 0.5 mg/mL of peptide samples for the CD investigation. For the FT-IR observation, the peptide solutions were freeze-dried at -42 ^{o}C and were analyzed using a FT-IR spectrophotometer. Before the measurements, the samples were grinded with potassium bromide (KBr) and pressed into pellets. The self-assembled morphologies of the peptide and their analogs were observed by transmission electronic microscopy (TEM, JEOL-2100, Japan). The TEM samples were prepared by dipping a copper grid with carbon film into the solution containing the self-assemblies. After the deposition, the samples were dried in air for one night. The negatively strained sample was prepared by dropping 5 μL of phosphotungstic acid strained solution onto the sample. Several minutes later, the residual solution was removed by filter paper and the sample was dried in air for one night.

4. Fluorescence spectroscopy

Fluorescence emission spectra of the suspended cells were recorded on a LS55 luminescence spectrometry (Perkin-Elmer) with excitation wavelength at 488 nm and the emission data was collected from 500 nm to 650 nm.

5. High-pressure liquid chromatography (HPLC)

The purity of the peptides was examined by high-pressure liquid chromatography (HPLC) with a C18 column according to the literature. The purity of P1 and P2 was 91.4% and 90.7%, respectively.

6. Cell culture

Human hepatocellular carcinoma (HepG2) cells, human cervix carcinoma (HeLa) cells, and human embryonic kidney transformed (293T) cells were incubated in DMEM medium with 10% FBS and 1% antibiotics of penicillin-streptomycin with 10^4 U/mL at 37 °C in a humidified atmosphere containing 5% CO_2, respectively.

7. Tumorous cell specific pre-targeting and fluorescent imaging

To evaluate the pre-targeting capacity of transferrin-biotin to tumorous cells, HepG2/HeLa/293T cells were firstly seeded into bio-housing chamber slide dishes loaded with a 25 mm diameter slide on cover-glass slides at a density of around 6×10^4 cells/well and cultured with 1 mL of 10% FBS-containing DMEM for 24 h at 37 ^oC prior to the addition of transferrin-biotin. After 1 mL of 0.5 mg/mL transferrin-biotin was added and co-cultured for 30 min at 4 ^oC, the medium was removed and the cells were washed with 200 μL of PBS for three times. HepG2/HeLa/293T cells without transferrin-biotin treatment were also cultured with PBS for 30 min at 4 ^oC. After the washing with PBS, each plate was incubated with 1 mL of PBS containing 6 μL of 1 mg/mL FITC-avidin for 30 min at 37 °C. Before the observation with confocal laser scanning microscopy (CLSM), all of the cells were washed three times with PBS to remove the residual FITC-avidin.

8. Evaluating the pre-targeting of transferrin-biotin to HepG2 cells by flow cytometry

The quantitative evaluation of tumorous cell pre-targeting was performed by flow cytometry (BD FACSAria TM III). HepG2 cells were seeded in 6-well plates (5×10^4 cells/well) and cultured in DMEM (1 mL) containing 10% FBS for 24 h. After the incubation, 1 mL of 0.5 mg/mL transferrin-biotin was added and co-cultured for 30 min at 4 ^{o}C. Then the medium was removed and the cells were washed three times with PBS. For the fluorescent labelling, 1 mL of PBS containing 6 μL of 1 mg/mL FITC-avidin was added and the cells were incubated for 30 min at 37 °C before the washing with PBS solution. The cells were then digested by trypsin and collected in centrifuge tubes by centrifuging at 1.2×10^4 rpm for 3 min. The supernatant was discarded and the left cells were washed twice with PBS. Then the suspended cells were filtrated and examined by flow cytometry. HepG2 cells without transferrin-biotin co-cultivation were used as negative control.

9. *In vitro* cytotoxicity assay

The cytotoxicity assay was performed with HepG2/HeLa/293T cells by MTT method. Briefly, the cells were seeded in 96-well plates at a density of 6×10^3 cells/well, and then cells were incubated in 100 μL of DMEM containing 10% FBS for 24 h prior to adding the peptide and their analogs. After the addition for 2 days, the medium was refreshed with 200 μL of fresh medium. Then 20 μL of MTT (5 mg/mL in PBS) solutions were injected to each well and further incubated for another 4 h. Then the medium was removed and 200 μL of DMSO was added. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability of peptides was calculated as: cell viability (%) = $(OD_{570\ (sample)}/OD_{570\ (control)})\times100$, in which $OD_{570\ (control)}$ and $OD_{570\ (sample)}$ were obtained in the

absence and presence of peptide materials, respectively. Each value was averaged from four independent experiments.

10. The targeted cytotoxicity

The targeted in vitro cytotoxicity assay of biotinylated peptide P3 against HepG2 cells was carried out. HepG2 cells were seeded in 24-well plates at a density of 2.5×10^4 cells/well, and then cells were incubated in 0.5 mL of DMEM containing 10% FBS for 1 day. 0.5 mL of 0.5 mg/mL transferrin-biotin was added and co-cultured for 30 min at 4 °C, the medium was removed and the cells were washed with 200 μL of PBS for three times. HepG2 cells without transferrin-biotin treatment were also cultured under same condition as control. After the washing with PBS, each plate was incubated with 1 mL of PBS containing 100 μL of 1 mg/mL avidin for 30 min at 37 °C. All of the cells were washed for three times with PBS prior to adding the peptides. Different concentration peptide solutions (20, 30, 40, 50, 60 μg/mL) were added into the cells. After the co-incubation for 2 days, the medium was refreshed with 200 μL of fresh medium. Then 50 μL of MTT (5 mg/mL in PBS) solutions were injected to each well and further incubated for another 4 h. Then the medium was removed and 500 μL of DMSO was added. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability of peptides was calculated as: cell viability (%) = $(OD_{570\ (sample)}/OD_{570\ (control)})\times100$, in which $OD_{570\ (control)}$ and $OD_{570\ (sample)}$ was obtained in the absence and presence of transferrin-biotin co-incubation, respectively. Each value was averaged from three independent experiments. The additional procedure of materials was shown in Fig. 5.

11. Confocal laser scanning microscopy for cell endocytosis

The tumorous cell line of HepG2 was seeded in a glass bottom dish at a density of 1×10^5 cells/well for 24 h. Thereafter, 1 mL of 0.5 mg/mL transferrin-biotin and 1 mL

of 10% FBS containing 100 μL of 1 mg/mL avidin were in turn added into the cells and co-cultured for 30 min at 4 ^{o}C and 37 ^{o}C, respectively. Then 10 μg/mL of RhB labeled peptide P4 was added and the cells were further incubated at 37 ^{o}C for 12 h and 24 h. After the incubation of HepG2 cells with peptide, the cells were further incubated with the fresh medium for another 12 h for the location of P4 at the mitochondria. Subsequently, 100 nM Mito Tracker Green FM was added and co-cultured for 30 min to stain the mitochondria. After washing with PBS for three times, the cells were observed under a laser scanning confocal microscopy (CLSM, Nikon C1-si TE2000, BD Laser). Meanwhile, HepG2 cells without transferrin-biotin incubation were also investigated under the same condition.

12. Investigation on the mitochondria damage-regulated apoptosis by JC-1 assay

HepG2 cells were cultured with 1 mL of 0.5 mg/mL transferrin-biotin for 30 min and 1 mL of 10% FBS containing 100 μL of 1 mg/mL avidin for another 30 min. Subsequently, the cells were incubated with 20 μg/mL of biotinylated peptide P4 at 37 °C for another 36 h. Then the cells mitochondria were stained with 10 μg/mL of JC-1 in DMEM for 30 min and washed with PBS for three times. The cells were observed with a laser scanning confocal microscopy. Meanwhile, HepG2 cells without transferrin-biotin incubation were also investigated under the same condition.

Supplementary Reference

S1. J. X. Chen, X. D. Xu, W. H. Chen and X. Z. Zhang. *ACS Appl. Mater. Interfaces*., 2014, **6**, 593.

S2. M. M. Javadpour,M. M. Juban,W. C. J. Lo, Steven M. Bishop, J. B. Alberty, S. M. Cowell, C. L. Becker and M. L. McLaughlin. *J. Med. Chem*., 1996, **39**, 3107.

Table S1. Sequences and molecular weights of KLA peptide and their amphiphiles (P1-4).

Peptide	Sequence	Theoretical molecular weight	Observed molecular weight
P1	$_{D}$(KLAKLAK)$_{2}$	1522.08	762.5, 1523.8 (ESI-MS)
P2	Fmoc-(ADDA)-Gly-$_{D}$(KLAKLAK)$_{2}$-Ala-Lys	2198.86	2198.16 (MALDI-TOF-MS)
P3	Fmoc-(ADDA)$_{2}$-Gly-$_{D}$(KLAKLAK)$_{2}$-Lys(biotin)	2551.40	2550.34 (MALDI-TOF-MS)
P4	RhB-(ADDA)$_{2}$-Gly-$_{D}$(KLAKLAK)$_{2}$-Lys(biotin)	2787.82	2752.40 (MALDI-TOF-MS)

Table S2. IC_{50} values of P1-4 to HeLa, HepG2 and 3T3 cell lines.

Peptide	HeLa	HepG2	3T3
P1	> 500 [a]	> 500	280
P2	36	50	55
P3	25	30	23
P4	28	32	35

[a] representing that the IC_{50} value is beyond the observed concentrations.

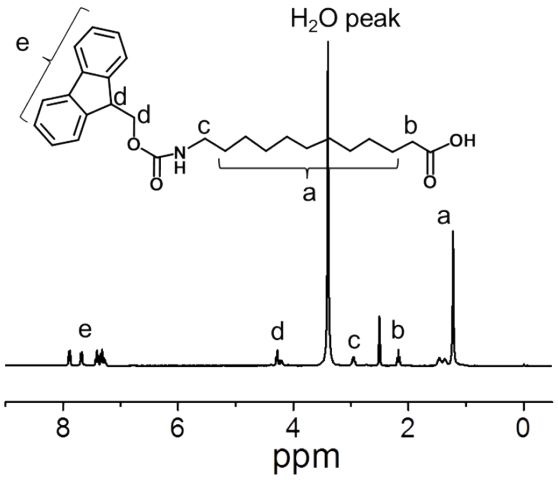

Fig. S1.[1]H NMR spectrum of Fmoc-C_{11}-COOH (ADDA) in DMSO-d6.

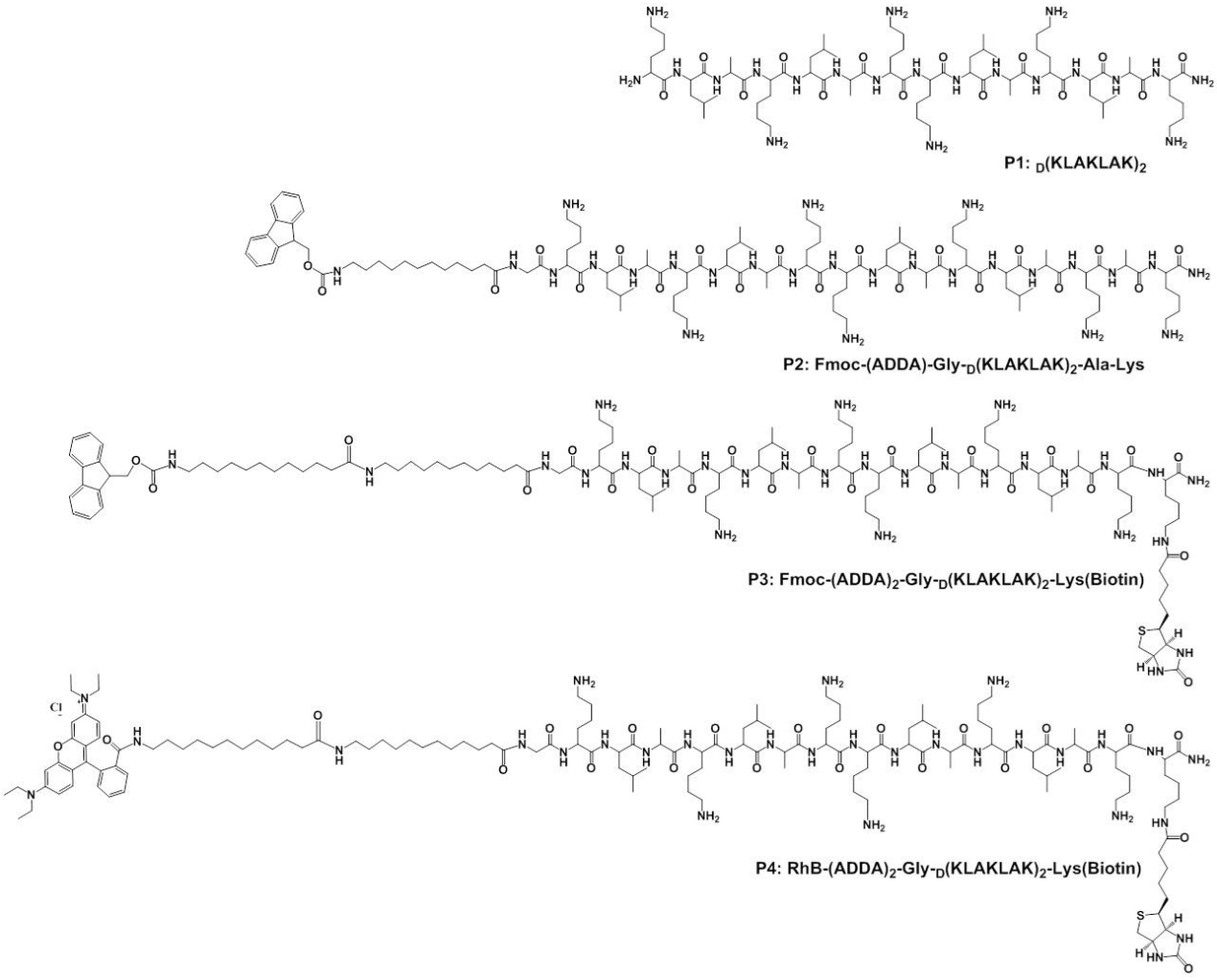

Fig. S2. The molecular structures of P1-4.

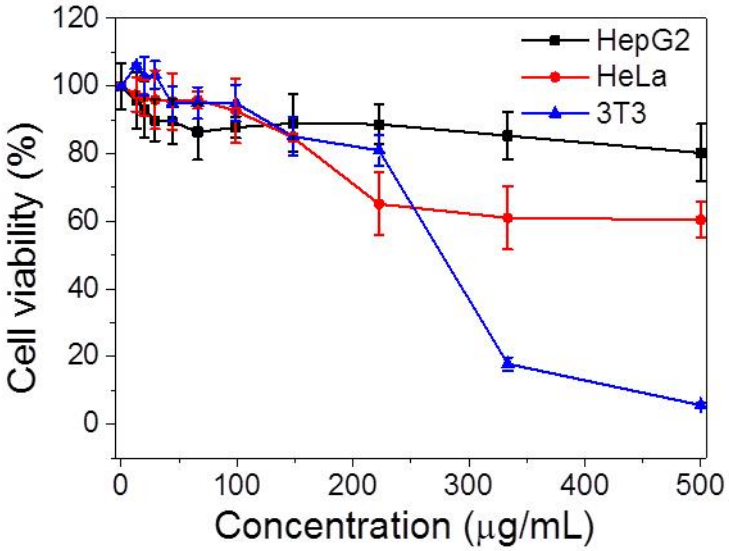

Fig. S3. The cell viability profiles of HepG2, HeLa and 3T3 cells after the co-incubation with P1 for 48 h.