Electronic Supplementary Information for:

A Novel Non-enzymatic Hydrolytic Probe for Dipeptidyl Peptidase IV Specific Recognition and Imaging

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1. Experimental

Reagents. Alogliptin, linagliptin and 7-aminoheptanoic acid were obtained from Bide Pharm., Ltd. (Shanghai, China). N,N-Diisopropylethylamine (DIPEA) were purchased from Acros Organics. O-(7-aza-1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU), acetic acid and 1-butylamine were purchased from Alfa Aesar Chemicals. 4-Bromo-1,8-naphthalic anhydride was purchased from Innochem Co. Ltd. (Beijing, China). Genistein, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), reduced glutathione (GSH), leucine aminopeptidase (LAP), esterase, prolidase, trypsin, dipeptidyl peptidase IV (DPP IV) were purchased from Sigma-Aldrich. RIPA (radio immunoprecipitation assay) lysis buffer (CW2333) was purchased from CWbiotech. Co. Ltd. (Beijing, China). Proteins were pure as judged by Coomassie-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE). DPPIV siRNA (sequences: GCACAGCACACCAACAUAUTTAUAUGUUGGUGUGCUGUGCTT), FAP siRNA (sequences: CGCCCUUCAAGAGUUCAUATTUAUGAACUCUUGAAGGGCGTT), poly(vinylidene fluoride)membranes (PVDF membranes), gel electrophoresis kits, western blot kits, enhanced chemiluminescence kits (ECL), Braford protein assay kits, Roswell Park Memorial Institute-1640 medium (RPMI-1640), calf serum (CS), MGC803 and KASE 30 cell lines were purchased from KeyGEN BioTECH Co. Ltd. (Nanjing, China). DPP IV, DPP VIII and DPP IX antibodies were purchased from ProteintechTM (USA). FAP antibody was purchased from Signalway Antibody, Inc. (USA). EntransterTM-R4000 transfection reagent was purchased from Engreen Biosystem Co. Ltd. (Beijing, China). Ultrapure water (over 18 MΩ•cm) from a Milli-Q reference system (Millipore) was used throughout. The commercial standard enzymatic DPP IV activity assay was purchased from RayBiotech (USA). The stock

solution (1.0 mM) of probe **1**, **2** or **FL** was prepared by dissolving requisite amount of it in DMSO. Stock solutions of other substances were prepared by dissolving in PBS or water.

Apparatus. Fluorescence measurements were made on a Fluoromax-4 Spectrofluorometer (France). UV-vis absorption spectra were measured in 1×1 cm quartz cells with a TU-1900 spectrophotometer (Beijing, China). ^{1}H NMR and ^{13}C NMR spectra were measured with a Bruker Avance 400 spectrometer. High resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded on a TripleTof 4600 instrument (AB Sciex, USA). Cell imaging experiments were operated on a TSC SPS-II confocal fluorescence microscope (Leica, Germany). Flow cytometer results were obtained from a BD LSRFortessa flow cytometry (USA). The western blot signal was detected using an ECL kit. The SDS-PAGE was operated with an electrophoresis apparatus (Jun Yi, Beijing, China). Cytotoxicity assay was made on a microplate absorbance reader (Biorad iMARKTM, USA). The fluorescence quantum yield (Φ) was determined by using a quantum efficiency measurement system: QE-2100 (Qtsuka Electronics Co. Ltd., Japan).

Synthesis of compound 1. The compound **1** was obtained according to the previous method (Wu et al. *Anal. Chem.* 2016, **88**, 1440).

Synthesis of FL. The compound FL, which was used to as the standard fluorophore for QY in this study, was obtained according to the previous method (Zou et al. *Biosens. Bioelectron.* 2017, **90**, 283).

Synthesis of compound 2. Compound **1** (331 mg, 1.0 mmol) was dissolved in 30 mL of 2-methoxyethanol, and 7-aminoheptanoic acid (217 mg, 1.5 mmol) was added into above solution. The reaction mixture was refluxed for 8 h. Then, the solvent was removed by evaporation under reduced pressure, and the residue as crude product was purified by silica gel chromatography eluted with CH_2Cl_2/methanol (v/v, 15/1), affording compound **2** as a yellow solid (198 mg, yield 50 %). The ^{1}H NMR and ^{13}C NMR spectra of compound 2 are shown below in Figure S1 and Figure S2, respectively. ^{1}H NMR (400 MHz, 298K, DMSO-d_6): δ 11.88 (s, 1H), 8.70-8.67 (d, J = 12 Hz, 1H), 8.40-8.38 (d, J = 8 Hz, 1H), 8.23-8.21 (d, J = 8 Hz, 1H), 7.73-7.71 (t, 1H), 7.65-7.62 (t, 1H), 6.73-6.71 (d, J = 8 Hz, 1H), 4.01-3.97 (t, 2H), 2.22-2.18 (t, 2H), 1.72-1.65 (m, 2H), 1.59-1.50 (m, 4H), 1.37-1.24 (m, 8H), 0.92-0.89 (t, 3H). ^{13}C NMR (100 MHz, 298K, DMSO-d_6): δ 174.98, 164.16, 163.31, 151.06, 134.66, 131.00, 129.85, 129.01, 124.54, 122.27, 120.54, 107.92, 104.11, 43.27, 34.14, 31.58, 30.29, 28.82, 28.17, 26.83, 24.94, 20.30, 14.19. HR-ESI-MS, calcd for $C_{23}H_{28}N_2O_4$ $[M+H]^+$: *m/z* 397.2127; found $[M+H]^+$: *m/z* 397.2080.

Synthesis of probe 1. The Compound **2** (99 mg, 0.25 mmol) was dissolved in 20 mL of CH_2Cl_2, HATU (143, 0.375 mmol) and DIPEA (82 μL, 0.375 mmol) were added, and the reaction mixture was stirred at 0 °C. After stirring for 40 min, alogliptin (85 mg, 0.25 mmol) was added into the above solution and the reaction was further stirred at room temperature for

6 h. Then, the mixture was diluted with CH_2Cl_2, and washed three times with water (100 mL×3). The solvent was removed by evaporation under reduced pressure, and the crude product was purified by silica gel chromatography eluted with CH_2Cl_2/methanol (v/v, 30/1), affording probe **1** as a yellow solid (54 mg, yield 30%). The ^{1}H NMR and ^{13}C NMR spectra of probe **1** are shown below in Figure S3 and Figure S4, respectively. ^{1}H NMR (400 MHz, 298K, DMSO-d_6): δ 8.69-8.67 (d, *J* = 8 Hz, 1H), 8.41-8.39 (d, *J* = 8 Hz, 1H), 8.24-8.22 (d, *J* = 8 Hz, 1H), 7.81-7.60 (m, 5H), 7.42-7.40 (d, *J* = 8 Hz, 1H), 7.23-7.21 (d, *J* = 8 Hz, 1H), 6.74-6.72 (d, *J* = 8 Hz, 1H), 5.33 (s, 1H), 5.13 (s, 2H), 4.01-3.97 (t, 2H), 3.71 (s, 1H), 3.06 (s, 4H), 2.96-2.93 (d, *J* = 12 Hz, 1H), 2.67 (s, 1H), 2.00 (s, 2H), 1.72-1.25 (m, 18H), 0.92-0.89 (t, 3H). ^{13}C NMR (100 MHz, 298K, DMSO-d_6): δ 172.18, 164.14, 163.30, 162.56, 159.86, 152.34, 151.03, 141.50, 134.62, 133.84, 133.47, 130.98, 129.83, 128.94, 128.27, 127.38, 124.52, 122.28, 120.52, 117.64, 110.45, 107.96, 104.99, 89.58, 55.39, 54.06, 51.45, 46.41, 45.28, 43.32, 35.67, 30.28, 29.39, 28.92, 28.19, 27.75, 26.86, 25.60, 23.16, 20.30, 14.18. HR-ESI-MS, calcd for $C_{41}H_{47}N_7O_5$ $[M+H]^+$: *m/z* 717.3717; found $[M+H]^+$: *m/z* 718.3714.

Synthesis of probe 2. Probe **2** was obtained according to method of probe **1**. The ^{1}H NMR and ^{13}C NMR spectra of probe **2** are shown below in Figure S5 and Figure S6, respectively. ^{1}H NMR (400 MHz, 298K, DMSO-d_6): δ 8.70-8.68 (d, *J* = 8 Hz, 1H), 8.42-8.41 (d, *J* = 4 Hz, 1H), 8.24-8.21 (d, *J* = 12 Hz, 2H), 7.91-7.64 (m, 6H), 6.74-6.72 (d, *J* = 8 Hz, 1H), 5.29 (s, 2H), 4.85 (s, 2H), 4.02-3.99 (t, 2H), 3.87 (s, 1H), 3.67-3.57 (m, 3H), 3.16-3.04 (m, 2H), 2.94-2.87 (t, 4H), 2.13-2.10 (t, 2H), 1.86-1.25 (m, 22H), 0.93-0.90 (t, 3H).). ^{13}C NMR (100 MHz, 298K, DMSO-d_6): δ 172.26, 169.23, 164.20, 163.35, 161.40, 156.22, 153.70, 151.33, 151.08, 149.51, 147.96, 134.64, 131.05, 129.86, 128.97, 128.32, 127.56, 126.15, 124.63, 122.95, 122.32, 120.56, 107.99, 104.17, 103.78, 81.66, 74.17, 55.34, 54.16, 50.26, 46.01, 45.42, 43.32, 35.76, 30.30, 29.84, 28.81, 28.17, 26.79, 25.61, 23.52, 22.00, 20.29, 18.57, 17.22, 14.18. HR-ESI-MS, calcd for $C_{48}H_{54}N_{10}O_5$ $[M+H]^+$: *m/z* 851.4357; found$[M+H]^+$: *m/z* 851.4339.

General procedures for spectroscopic measurements. Unless otherwise stated, all the fluorescence measurements were made according to the following procedure. In a test tube, 10 μL of stock solution of probe **1** and appropriate volume of PBS were mixed, followed by adding an appropriate volume of the solution of DPP IV or other substances. The mixed solution was adjusted to 2 mL with PBS. After incubation at 37 °C for 40 min, the reaction solution was transferred to a quartz cell of 1-cm optical length to measure fluorescence with $\lambda_{ex/em}$ = 450/550 nm (both excitation and emission slit widths were set to 2.5 nm). For pH and temperature texts, the same method was used while no DPP IV or other substances were added. For absorbance measurements, 10 μL of stock solution of probe **1**, **2** or **FL** and appropriate volume of PBS were mixed and the volume of mixed solution was 2 mL (No DPP IV and other substances were added).

Cell imaging and flow cytometer experiment. MGC 803 cells (including all kinds of cells used in this study) were cultured for imaging. Before imaging, the culture media were removed, and the cells were washed using RPMI-1640 for three times. Then, the cells were incubated with probe **1** (5 μM) or probe **2** (5 μM) at 37 °C for appropriate time in RPMI-1640, washed with RPMI-1640 three times to remove the free probe, and subjected to fluorescence imaging experiments or were preserved in PBS to flow cytometer experiment.

Immunohistochemical (IHC) assay. IHC images were obtained according to the previous method (Wu et al. *J. Am. Chem. Soc.* 2015, **137**, 2366) in KeyGEN BioTECH Co. Ltd. (Nanjing, China). The brown area in images represents the expression of DPP IV.

Transfection. The siRNA-transfected MGC803 cells were obtained according to the following procedure. MGC803 cells were cultured at 37 °C for one day before transfection. The corresponding siRNA at a concentration of 100 nM was prepared by dissolving in RPMI-1640 and 5 μL of Entranster^{TM}-R4000 transfection reagent. The mixture was added to Petri dishes, and the cells were cultured at 37 °C for 6 h. Then the culture medium was discarded, and the fresh RPMI-1640 medium was added. The cells were incubated at 37 °C for 48 h for further use.

Western blot. The cell lysates were prepared according to the previous method (Gong et al. *Chem. Sci.* 2016, **7**, 788). The western blot analyses were made according to the previous method (Li et al. *Angew. Chem. Int. Ed.* 2015, **54**, 10821). Note that glyceraldehyde-3-phosphate dehydrogenase, GAPDH, was used as a protein standard.

Cytotoxicity Assay. MGC 803 cells were cultured in RPMI-1640 and supplemented with 10% CS. The cells were incubated at 37 °C with 5% CO_2. The cytotoxicity of probe **1** was tested on cells using a standard MTT assay, as described previously (Ren et al. *Nanoscale* 2017, **9**, 11195).

Measurement of IC_{50} value. First, 50 pg/mL of DPP IV were incubated with different concentrations of probe **1** or probe **2** at 37 °C for 60 min. And then the enzymatic activities of DPP IV of different samples were measured using the commercial standard enzymatic DPP IV activity assay according to the instructions. The IC_{50} values were calculated according to the fitting curves.

Statistical tests. The *t* analysis was operated according to the previous method (Gong et al. *Chem. Sci.* 2016, **7**, 788).

2. Supporting Figures

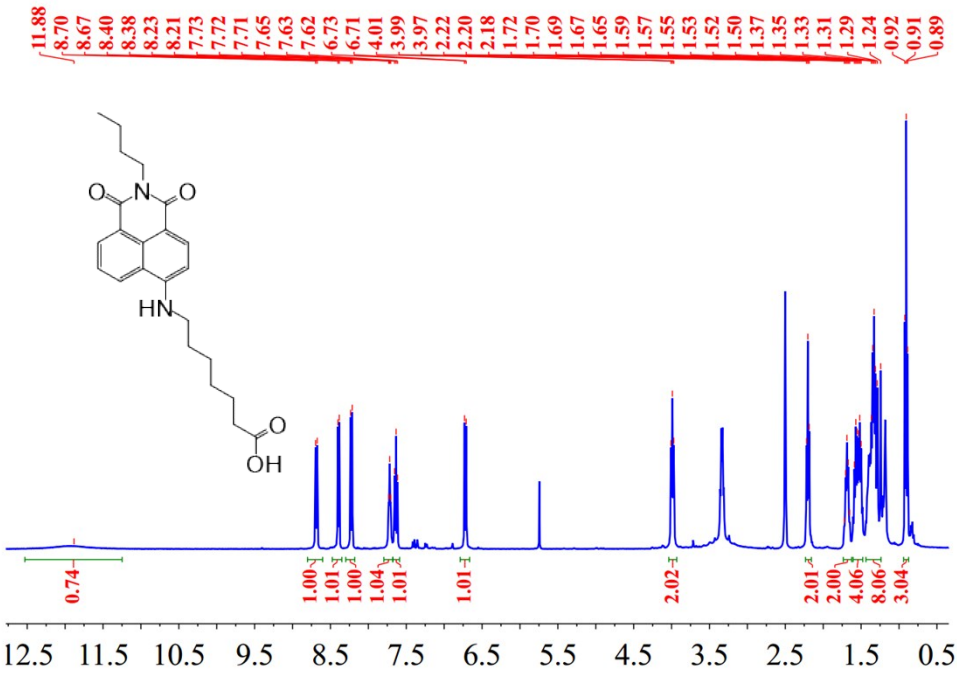

Figure S1. ^{1}H NMR spectrum of compound **2** (400 MHz, DMSO-d_6, 298 K).

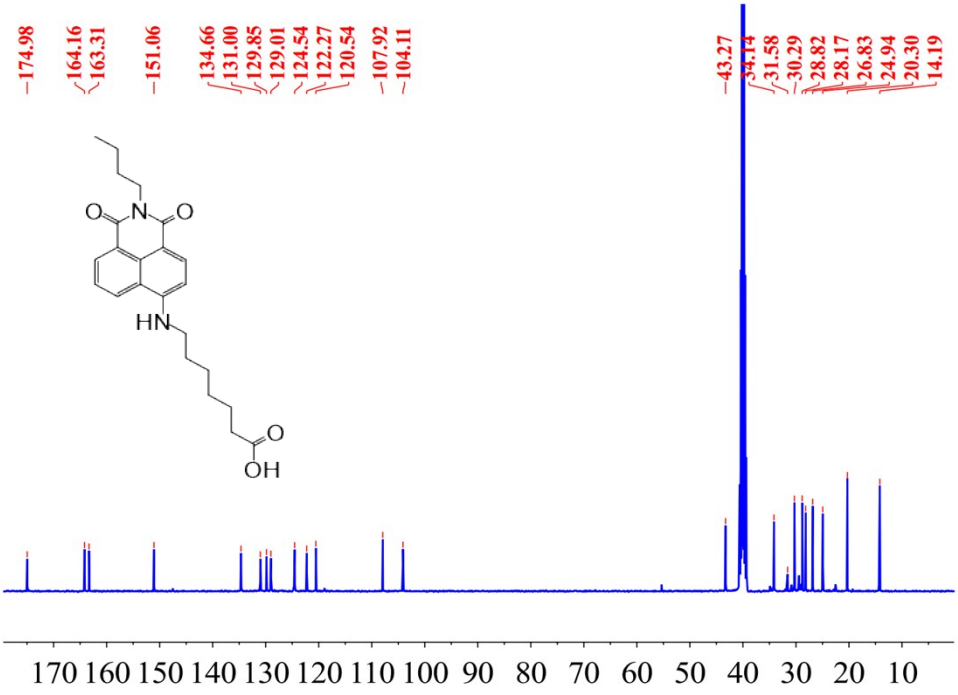

Figure S2. ^{13}C NMR spectrum of compound **2** (100 MHz, DMSO-d_6, 298 K).

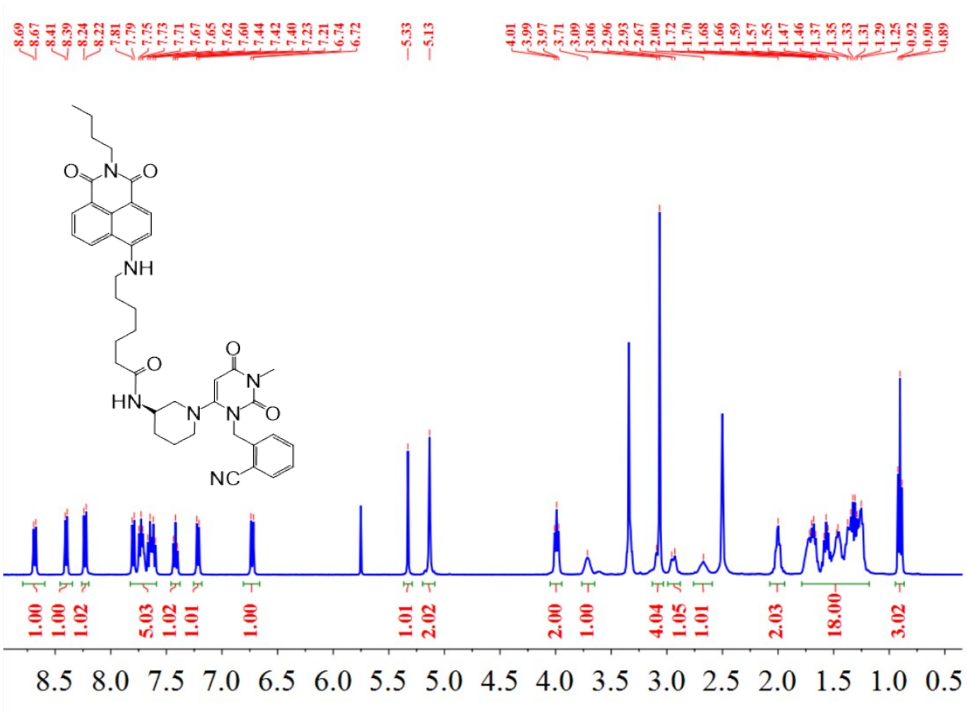

Figure S3. ^{1}H NMR spectrum of probe **1** (400 MHz, DMSO-d_6, 298 K).

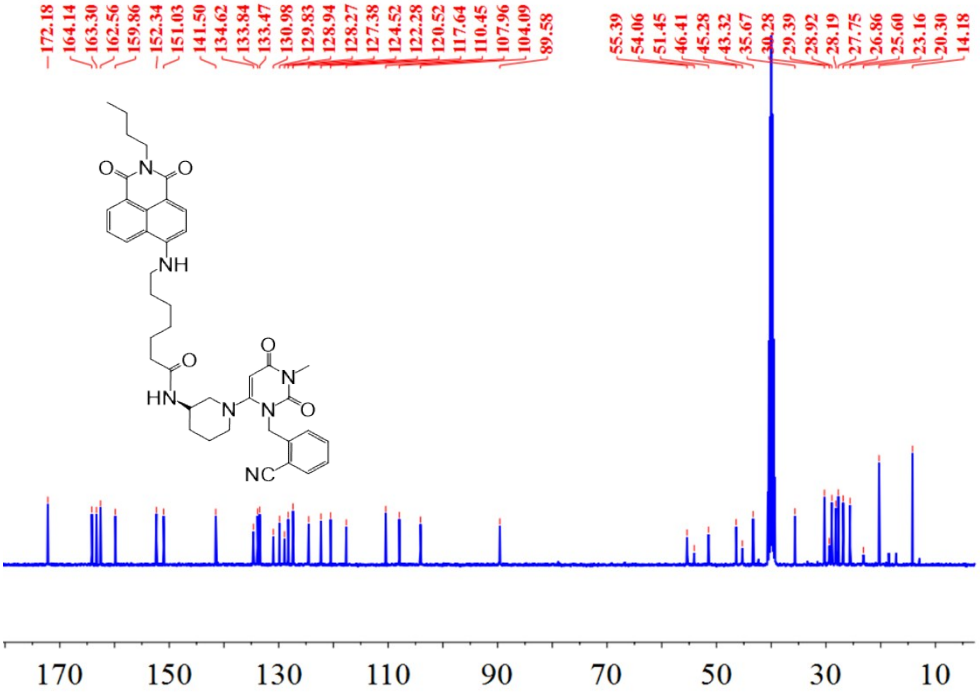

Figure S4. ^{13}C NMR spectrum of probe **1** (100 MHz, DMSO-d_6, 298 K).

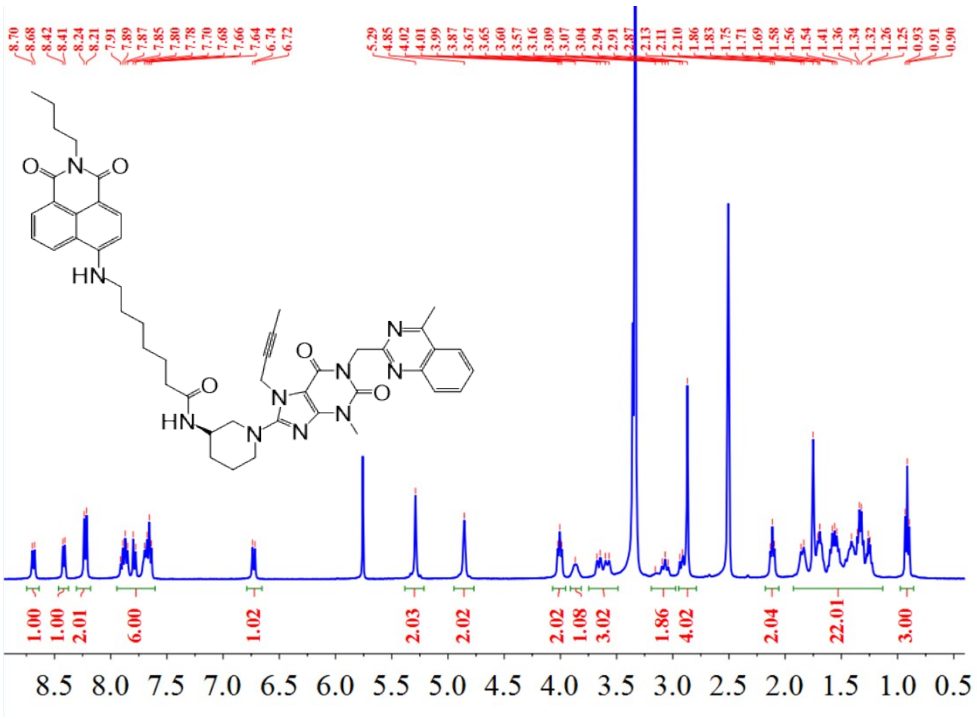

Figure S5. ^{1}H NMR spectrum of probe **2** (400 MHz, DMSO-d_6, 298 K).

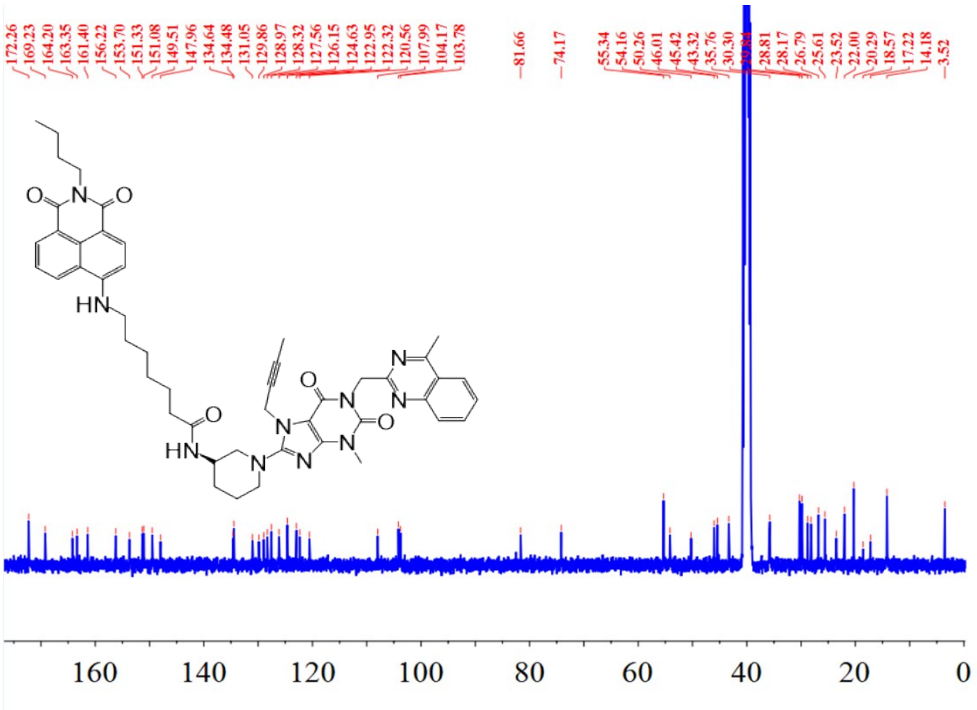

Figure S6. ^{13}C NMR spectrum of probe **2** (100 MHz, DMSO-d6, 298 K).

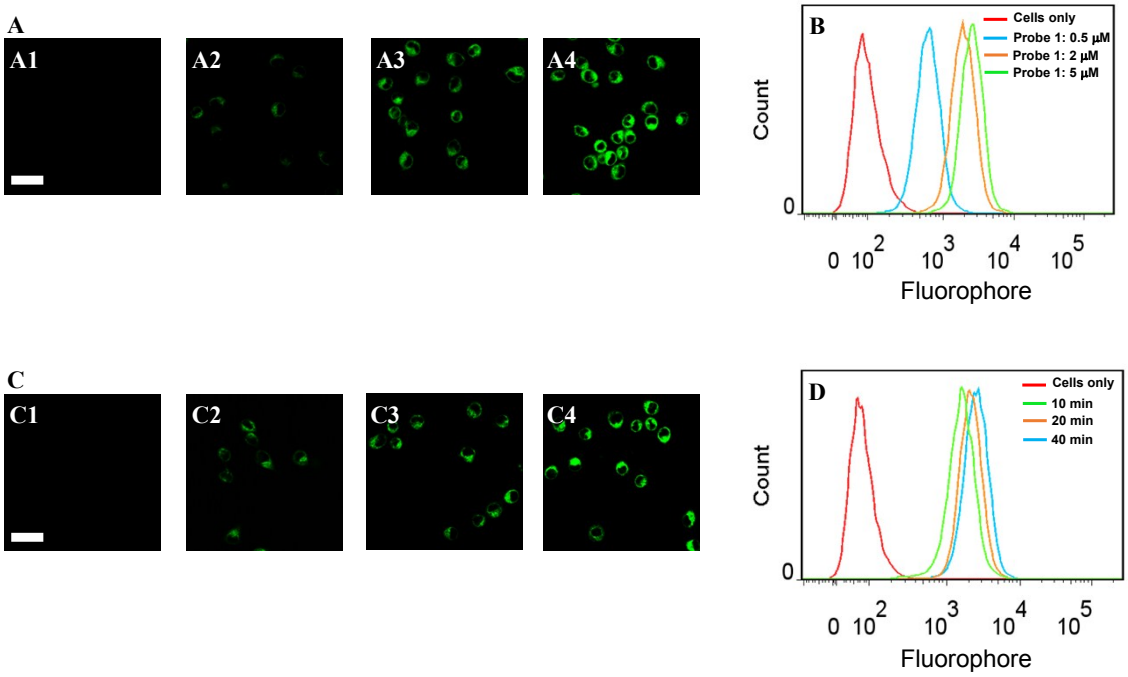

Figure S7. (A) The effect of concentration of probe **1** on the fluorescence of MGC 803 cells. (A1) Cells only; (A2-A4) Cells incubated with 0.5 μM, 2 μM and 5 μM of probe **1** for 40 min, respectively. (B) The flow cytometry results of above cells. (C) The effect of incubation time of probe **1** on the fluorescence of cells. (C1) Cells only; (C2-C4) Cells incubated with 5 of probe **1** for 10, 20, 40 min, respectively. (D) The flow cytometry results of above cells. Scale bar 50 μm.

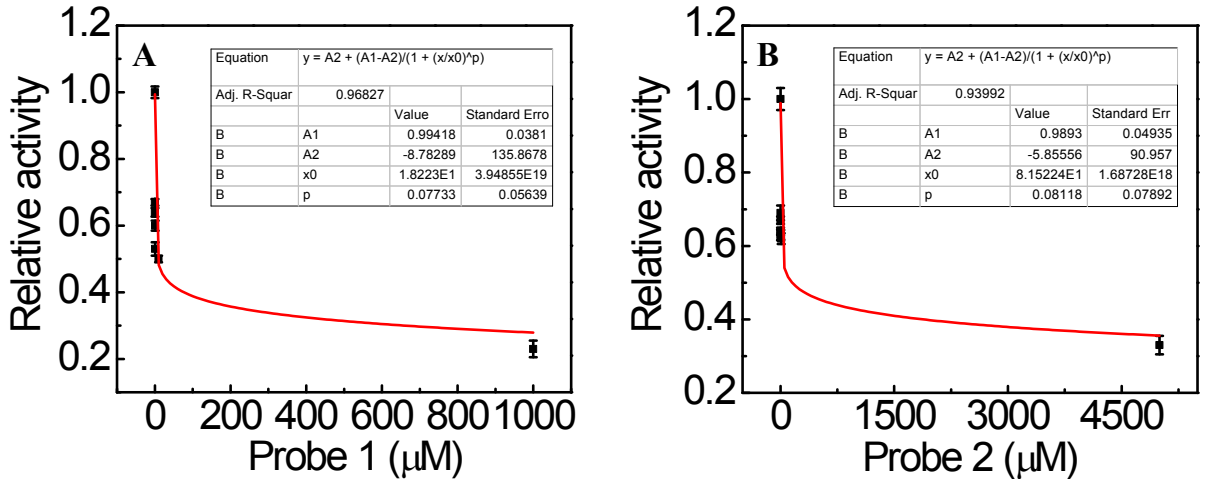

Figure S8. The relative enzymatic activity of DPP IV incubated with different concentrations of probe **1** (A) and probe **2** (B) measured by the commercial standard enzymatic activity assay. The enzymatic activity of untreated DPP IV is defined as 1.

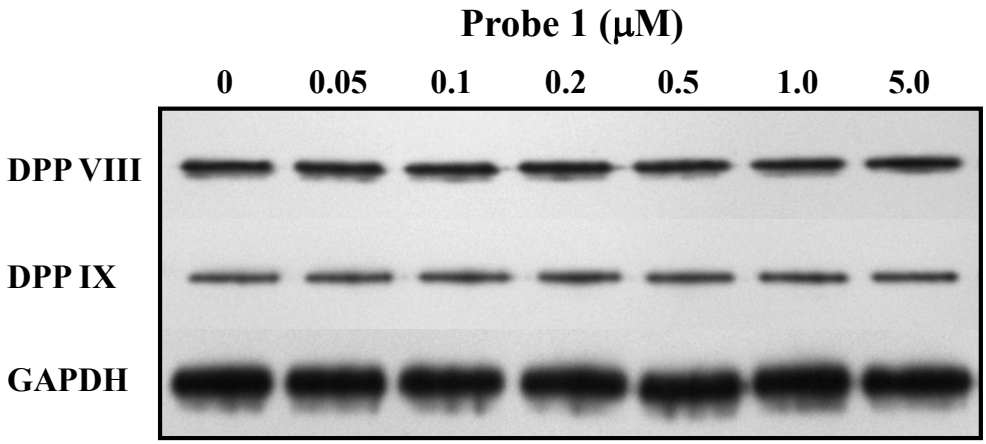

Figure S9. Western blot results of cells incubated with probe **1** for 40 min.

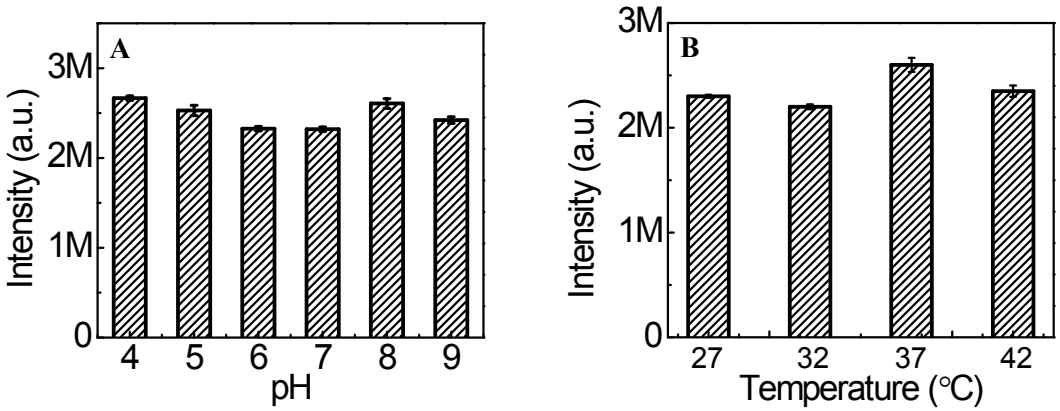

Figure S10. Effects of (A) pH and (B) incubation temperature on the fluorescence of probe **1** (5 μM). Conditions: (A) the probe was incubated in different pH for 40 min. (B) the probe was incubated in 10 mM phosphate buffer (pH 7.4) at different temperature for 40 min.

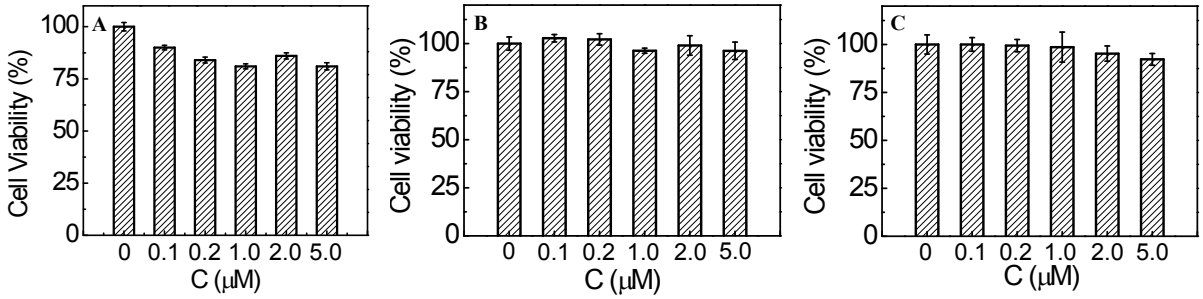

Figure S11. Effects of probe **1** at varied concentrations on the cell viability of (A) MGC803 cells; (B) K 30 cells; (C) MCF-7 cells. The cell viability without the probe is defined as 100%.

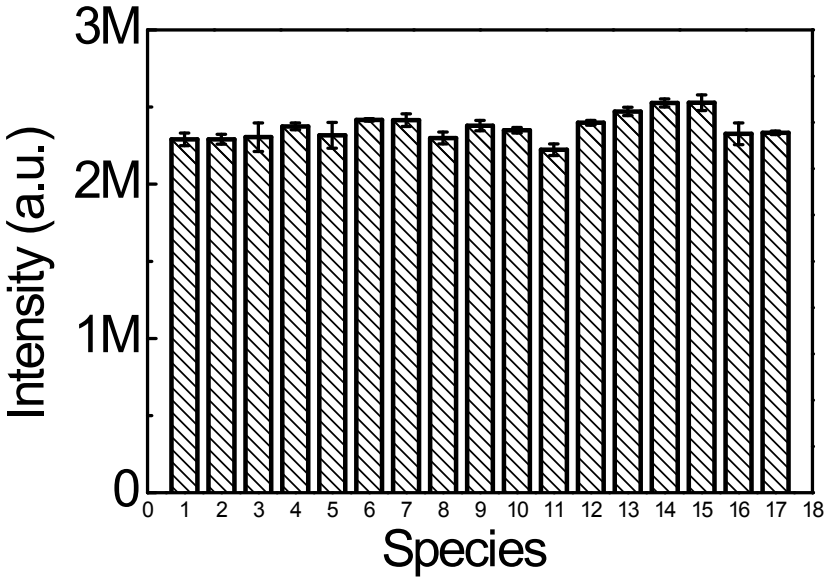

Figure S12. Fluorescence change of probe **1** (5 μM) in the presence of various species: (1) probe only; (2) 150 mM KCl; (3) 2.5 mM $CaCl_2$; (4) 2.5 mM $MgCl_2$; (5) 100 μM $CuCl_2$; (6) 10 mM glucose; (7) 1 mM Lysine; (8) 1 mM Cysteine; (9) 1 mM Threonine; (10) 1 mM Phenylalanine; (11) 1 mM glycine; (12) 5 mM GSH; (13) 90 μg/mL esterase; (14) 250 μg/mL trypsin; (15) 25 μg/mL prolidase; (16) 30 μg/mL LAP; (17) 1 μg/mL DPP IV.

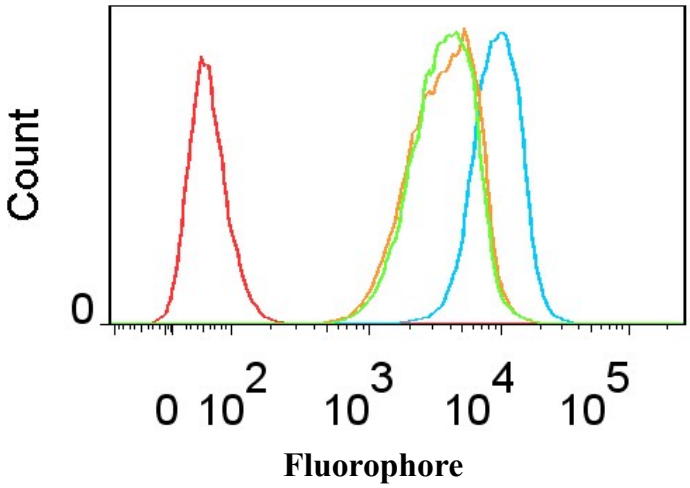

Figure S13. The flow cytometry results of figure 3E (red: cells only; blue: cells incubated with probe **1** (5 μM) for 40 min; green: cells incubated with probe **1** (5 μM) for 40 min first and then alogliptin (1 μM); brown: cells incubated with probe **1** (5 μM) for 40 min first and then alogliptin (5 μM).

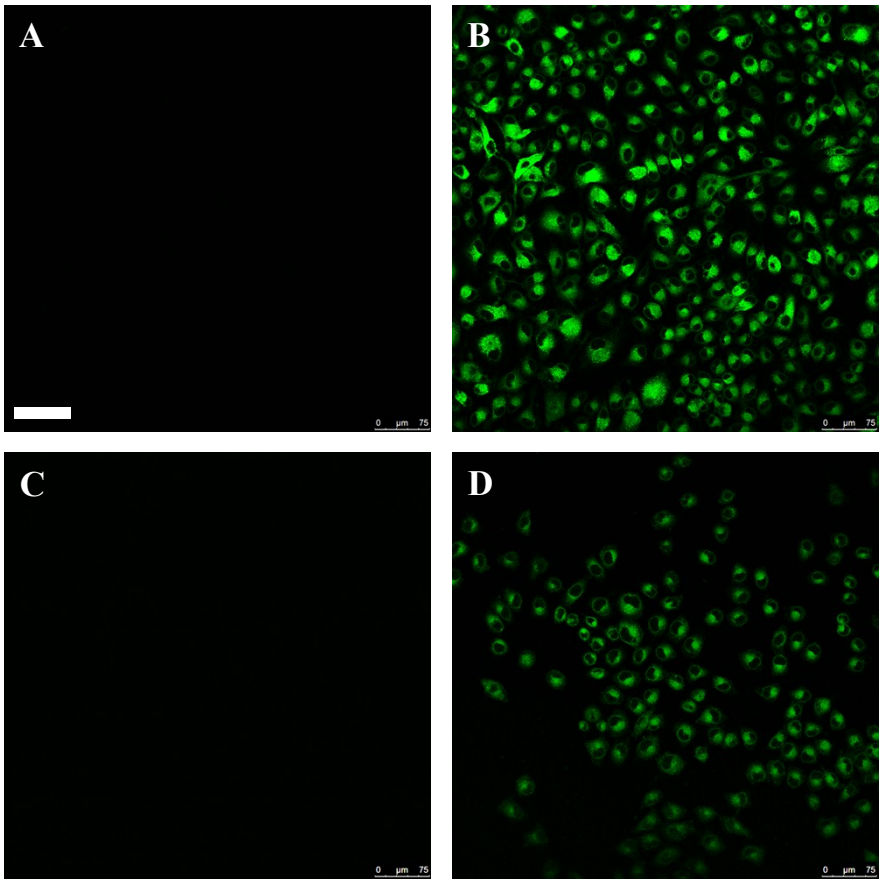

Figure S14. The fluorescent images of A549 and MCF-7 cells. (A) A549 cells only; (B) A549 cells incubated with probe **1** (5 μM) for 40 min; (C) MCF-7 cells only; (D) MCF-7 cells incubated with probe **1** (5 μM) for 40 min. Scale bar 75 μm.