Supplementary Information for

Fluorescent switchable AIE probe for selective imaging of dipeptidyl peptidase-4 *in vitro* and *in vivo* and its application in screening DPP-4 inhibitors

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Experimental details

Materials

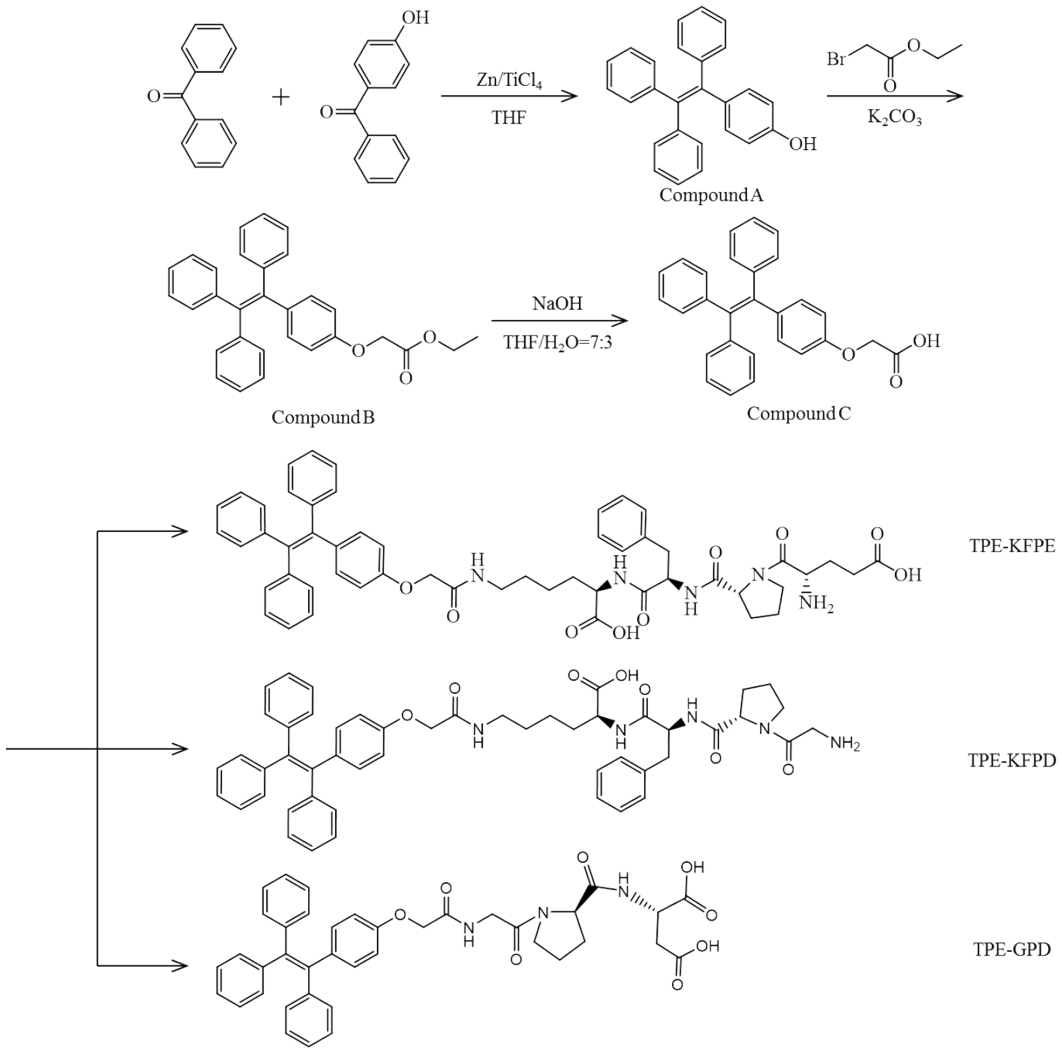
DPP-4, diprotin A, cytochrome C (CYC), bovine serum albumin (BSA), α-chymotrysin and lysozyme were purchased from Sigma-Aldrich (USA). Trypsin was purchased from Solarbio (China). DPP-8 was purchased from Cloud-Clone (USA). Human serum albumin (HSA) was purchased from TCI (Japan). Collagenase type I, II and bovine serum (BS) were purchased from Gibco (USA). DMEM medium was purchased from Corning Incorporated Life Sciences (USA). Proteinase K, benzophenone, 4-hydroxybenzophenone, TiCl4, tert-butyl bromoacetate, K_2CO_3 were purchased from Aladdin. Solid phase synthesis of TPE-Lys-Phe-Pro-Glu (TPE-KFPE), TPE-Lys-Phe-Pro-Gly(TPE-KFPG), and TPE-Gly-Pro-Asp(TPE-GPD) were conducted by Shanghai Top-peptide Bio (China). Other reagents without being listed were used as received.

Synthesis of fluorescent probes

First, benzophenone (2.2 g, 12 mmol), 4-Hydroxybenzophenone (1.9 g, 10 mmol), zinc powder (2.9 g, 44 mmol) and tetrahydrofuran (80 mL) were mixed using magnetic stirrer in a three-necked flask, under an N_2 atmosphere. $TiCl_4$ (2.4 mL, 22 mmol) was slowly added drop by drop during stiring at 0 °C. The mixture was suffered reflux overnight at 85℃. 200 mL CH_2Cl_2 and 200 mL HCl were added to the mixture and the organic part of the mixture was extracted. And the solution was dehydrated by $MgSO_4$, and the solution was removed by rotary evaporator. Then, the crude product was purified by silica gel column and white solid (Compound A) was got. Compound A (1 g), ethyl bromoacetate (0.576 g), K_2CO_3 (0.5 g) and acetonitrile were added into a flask. The mixture was reflux at 110°C for 24 h. After filtration, this product was purified by silica gel column and get compound B. Compound B (0.5 g) was dissolved by tetrahydrofuran (28 mL) with 12 mL NaOH solution (4.17 M) and stirred. After 24 h, through extraction and vacuum distillation, white solid powder was obtained, which was TPE-COOH (Compound C). TPE-KFPE, TPE-KFPG, TPE-GPD were synthesized through solid phase synthesis.

To obtain product of enzymatic reaction, DPP-4 was incubated with TPE-KFPE(10 mg) at 37℃. After 48 h, we got the reaction products of TPE-KFPE reaction with DPP-4 by centrifugal freeze-drying.

The ^1H NMR specta of TPE-KFPE and the product of enzymatic reaction after incubated with DPP-4 were shown in Fig.S2. The ^{13}C NMR spectrum of TPE-KFPE was shown in Fig.S3. The mass spectra of TPE-KFPE and the product of enzymatic reaction after incubated with DPP-4 were shown in Fig.S7. The HPLC chromatogram of TPE-KFPE was shown as and Fig.S4.



Scheme S1 Detailed synthetic route of TPE-KFPE, TPE-KFPG, TPE-GPD.

The Choices of fluorescent probes

DPP-4 (5 mU/mL) was incubated with TPE-KFPE, TPE-GPD (10 μM) respectively for 30 min at 37℃, while DPP-4 (10 mU/mL) was incubated with TPE-KFPG (20 μM). The PL spectra were measured in the range from 400 to 600 nm with JASCO FP-6500 spectrophotometer (JASCO, Japan), while the excitation wavelength was 320 nm. As shown in Fig.S1, after incubation with DPP-4, only the PL intensity of TPE-KFPE increased. Finally, TPE-KFPE was chosen as the fluorescent probe.

TPE-KFPE fluorescence detection

The TPE-COOH and TPE-KFPE, diluted in HEPES (0.5 mM, pH 7.0), were

prepared with 10 μM respectively. The PL spectra were measured in the range from 400 to 600 nm with JASCO FP-6500 spectrophotometer, while the excitation wavelength was set at 320 nm.

Enzymatic fluorescence and diprotin A inhibition

To ensure the enzymatic reaction of DPP-4 hydrolyzing the probe, 5 mU/mL DPP-4 was incubated with 10 μM TPE-KFPE for 30 min at 37℃ in the presence or absence of inhibitor diprotin A (5μM). For LC-MS analysis, 10 mU/mL DPP-4 was incubated with 50 μM TPE-KFPE for 30 min at 37℃ in the presence or absence of inhibitor diprotin A (5μM). The PL spectra were measured from 400 to 600 nm with JASCO FP-6500 spectrophotometer (JASCO, Japan), while the excitation wavelength was set at 320 nm. Finnigan LCQ Deca XP^{plus} ion trap mass spectrometer (Thermo Finnigan, USA) was used for LC-MS analysis. The parameters of LC-MS analysis were as follows: nebulizing gas, high purity nitrogen (N_2); collosion gas, high-purity helium (He); ion spray voltage: -3 kV; capillary temperature: 350℃; capillary voltage: −15 V; mass range: *m/z* 100−1500. The LC-MS method was applied with a reversed-phase Zorbax SB-C_{18} analytical column (250 mm × 4.6 mm I.D., 5 μm, Agilent Technologies, USA) by a gradient elution using 0.1% (v/v) formic acid (A) – acetonitrile (B) as the mobile phase 0 min (70% A)→5 min (70% A)→35 min (25% A)→45 min (5% A). The flow rate was 0.6 mL/min.

Optimal pH of HEPES buffer

In order to determine the optimal pH of HEPES buffer (0.5 mM), DPP-4 (5 mU/mL) was incubated with TPE-KFPE (10 μM) for 30 min at 37℃ in HEPES buffer with pH 4.0, 6.5, 7.0, 8.2, and 10.0 respectively. The PL intensity was immediately measured by TECAN Infinite F200 Multi-function microplate (Tecan, Austria) with the parameters: excitation wavelength 320 nm, emission wavelength 450 nm. As shown in Fig.S5, the acidified (pH 4.0) or alkalized solution (pH 10.0) led to the

invalid assay. The solution with pH range of 6.5 to 8.2 can produce appropriate fluorescent signal.

Optimal concentration of HEPES buffer

To explore the optimal concentration of HEPES buffer, DPP-4 (5 mU/mL) was incubated with TPE-KFPE (10 μM) for 30 min at 37℃ with different concentration of HEPES buffer (pH 7.0). The concentration of HEPES buffer was ranged from 0.1 mM to 50 mM. The PL intensity was performed using TECAN Infinite F200 multifunction microplate reader with an excitation wavelength of 320 nm and an emission wavelength of 450 nm.

Enzyme kinetics assay

TPE-KFPE (10 μM) was incubated with/without DPP-4 (5 mM/mL) at 37℃. The PL intensity was immediately measured by TECAN infinite F200 Multi-function microplate (excitation wavelength 320 nm, emission wavelength 450 nm). In the presence of DPP-4, the PL intensity was gradually increasing by the time until the PL intensity was stable.

Dose-dependent DPP-4 assay

In order to choose the appropriate concentration of DPP-4 in the assay, TPE-KFPE (10 μM) was incubated with different concentrations of DPP-4 (from 0.1 mU/mL to 20 mU/mL) at 37℃ for 30 min. As shown in Fig.2B, with the increasing concentration of DPP-4, the PL intensity was gradually increased and more fluorescent hydrolysate TPE-KF was released. The PL intensity was measured by TECAN infinite F200 Multi-function microplate (excitation wavelength 320 nm, emission wavelength 450 nm).

To investigate the linearity of DPP-4 in 3T3-L1 cells, 3T3-L1 (2×10^6 cells) cells were seeded in culture dish. After 24 h, cells were washed with HEPES buffer. Cell protein was prepared from 3T3-L1 by repeated freeze thaw method. The protein

content was determined by BCA protein quantitative method. 3T3-L1 cell protein with different concentration (0.414，0.976，4.60，8.78，21.3，41.4，82.8 ng/μL) and TPE-KFPE (10 μM) were incubated for 30 min at 37 ℃. The PL intensity was measured by TECAN infinite F200 multifunction microplate reader (λ_{ex}=320 nm, λ_{em}=450 nm).

Dose-dependent inhibition of DPP-4 by diprotin A

The DPP-4 (5 mU/mL) and TPE-KFPE (10 μM) were incubated with different concentration of diprotin A at 37℃ for 30 min. The concentration of diprotin A was ranged from 0.005 μM to 100 μM. The PL intensity was recorded by TECAN infinite F200 multi-function microplate with the same parameters as dose-dependent DPP-4 assay.

Enzymatic specificity

In order to investigate the specificity of the fluorescent probe TPE-KFPE to recognize DPP-4, TPE-KFPE (10 μM) was incubated with DPP-4 (5 mU/mL) and other enzymes or proteins at 37℃ for 30 min. Human serum albumin (HSA), bovine serum albumin (BSA), collagenase I/II (Coll I/II), cytochrome C (CYC), lysozyme, trypsin, proteinase K, DPP-8, and α-chymotrysin were chosen as the comparison samples. The PL intensity was performed using TECAN infinite F200 multifunction microplate reader with an excitation wavelength of 320 nm and an emission wavelength of 450 nm.

Dose-dependent inhibition of DPP-4 by Epigallocatechin Gallate

Epigallocatechin gallate (EGCG) was found in our primary screening as a DPP-4 inhibitor. EGCG with different concentration(5, 10, 25, 50, 100 μM) was incubated with DPP-4 (5 mU/mL) and TPE-KFPE (10 μM) for 30 min at 37 ℃. The PL intensity was measured in the condition of excitation wavelength 320nm and emission wavelength 450nm on TECAN infinite F200.

Cell culture

3T3-L1 preadipocytes were obtained from Cell bank of Chinese Science Academy, Shanghai, China. 3T3-L1 preadipocytes were grown in DMEM (Corning, USA) with 10% bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, USA). Cells were cultured in 5% humidified CO_2 atmosphere at 37℃.

Cytotoxicity of TPE-KFPE

3T3-L1 (4×10^3 cells/well) cells seeded in 96-well plates were treated with respective concentrations of TPE-KFPE (10, 30, 50 μM) for 24 h. Cell survival rate was measured by MTT assay. Absorbance was measured at 580 nm using TECAN Infinite F200 multifunction microplate reader. As shown in Fig.S9, TPE-KFPE showed no cytotoxic effect on 3T3-L1 cells.

$$\text{Cell survival}(\%) = \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100\%$$

Living cell imaging of DPP-4 activity in 3T3-L1 cells using TPE-KFPE

3T3-L1 (1×10^5 cells/well) cells were seeded in confocal culture dish. After 24 h, cells were pre-incubated with diprotin A (0.1 mM) for 15 min. Then, the medium was subsequently replaced by medium contained TPE-KFPE (50 μM) for 1 h. After incubation, the cells were washed with PBS. Fluorescence images were captured with 60× lens by Nikon A1R laser scanning confocal microscope equipped with 405nm laser. The fluorescent intensity was calculated by software Image-J, and summed the total fluorescent intensity of cells from six images.

***In vivo* imaging of DPP-4 in zebrafish using TPE-KFPE**

The zebrafish were chosen 6-7 days post fertilization. Wild type zebrafish was bred in sea salt water with 1-phenyl-2-thiourea (PTU) at 28.5 ºC. One group of zebrafish was bred with diprotin A (1 mM) for 24 h. The zebrafish were microinjected with TPE-KFPE (2.5 mM, 10 nL). After 2 h, the fluorescent images were taken by

Leica DMI6000B fluorescent microscope with Andor Zyla cMOS camera (5× lens). The relative fluorescent intensity was calculated by Image-J. The mean fluorescent intensity of 6 different samples in each group was calculated. Fluorescent images were also captured by Nikon A1R laser scanning confocal microscope equipped with 405 nm laser. Optical slices through islet were acquired by moving focal plane (x,y) along z-axis from the bottom to the top of the islet at 2-μm increments.

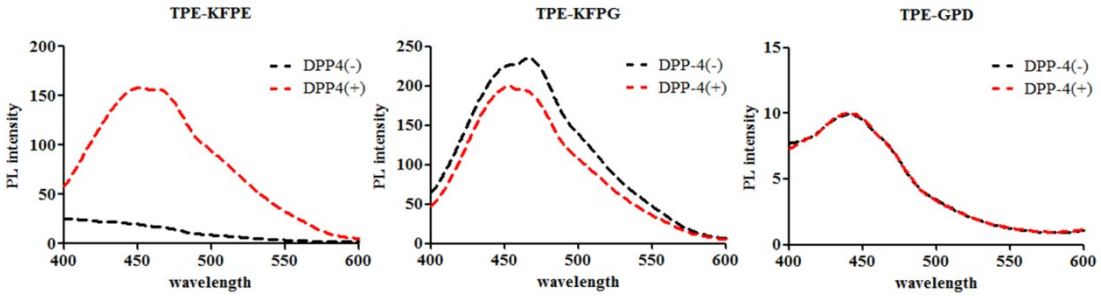


Figure S1 PL spectra of different TPE-based probes with DPP-4

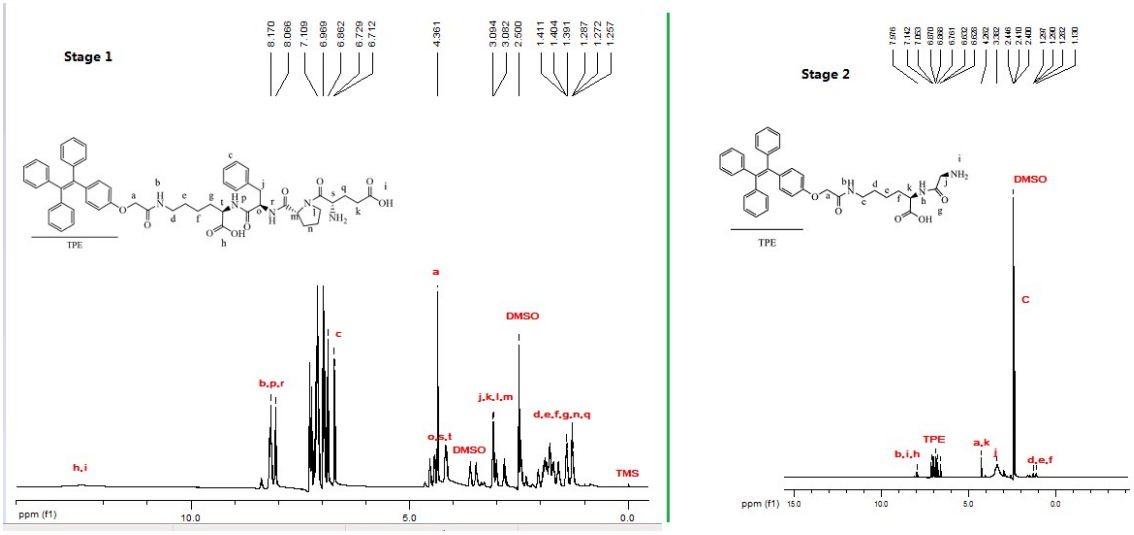


Figure S2 ^{1}H NMR spectra of TPE-KFPE (Stage 1) and the probe after incubation with DPP-4 (Stage 2)

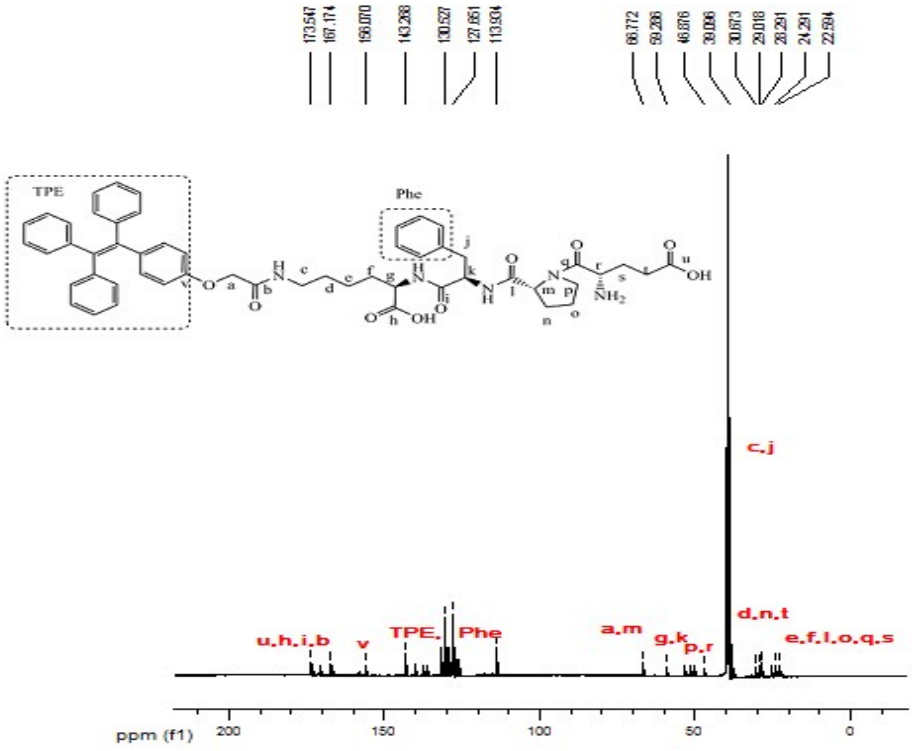


Figure S3 ^{13}C NMR spectrum of TPE-KFPE

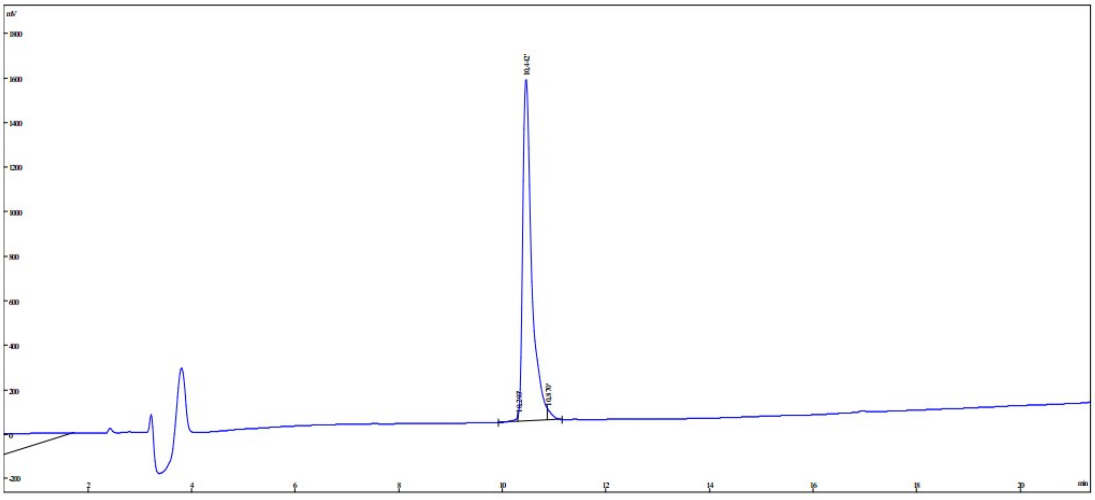


Figure S4 HPLC chromatogram of TPE-KFPE

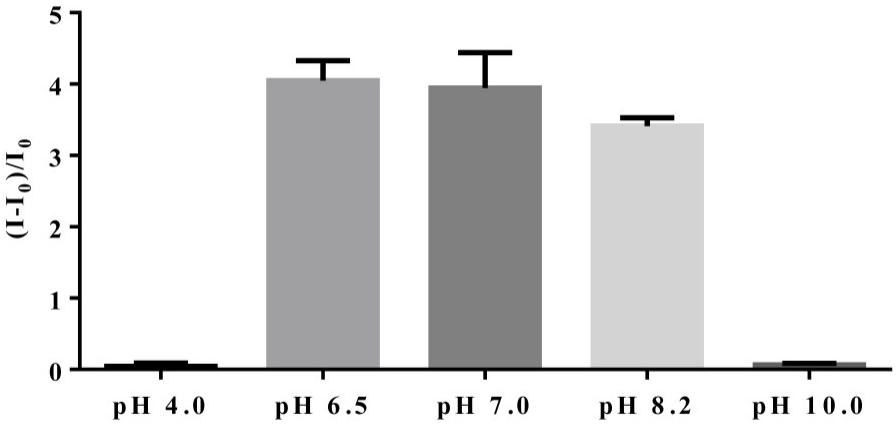


Figure S5 The $(I-I_0)/I_0$ value of probe incubated with DPP-4 in HEPES buffer with different pH value.

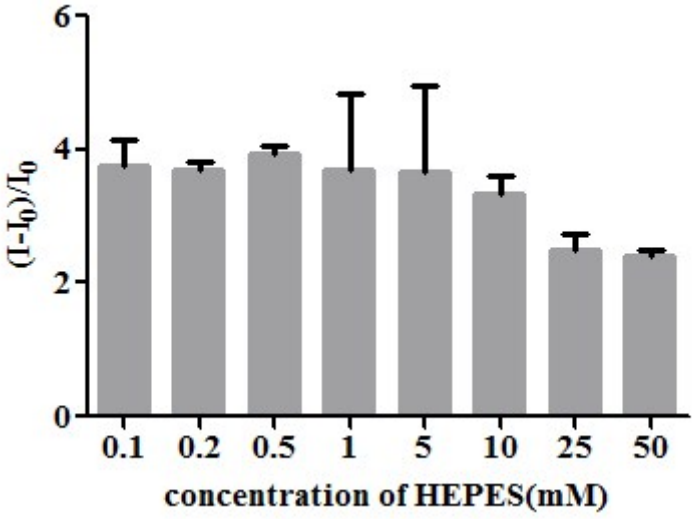


Figure S6 The $(I-I_0)/I_0$ value of probe incubated with DPP-4 in HEPES buffer with different concentration.

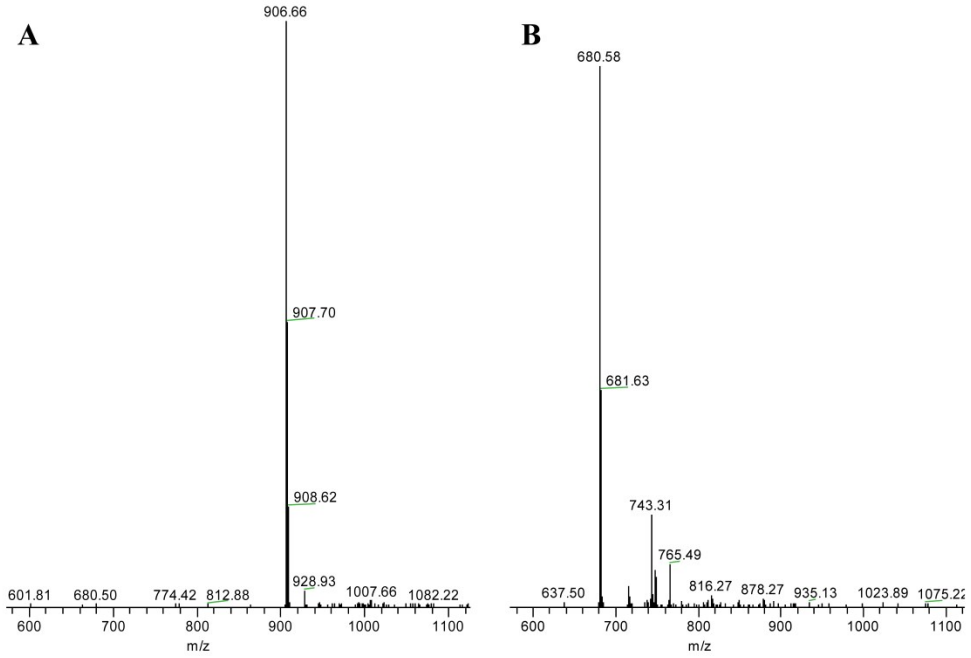


Figure S7 Mass spectra of TPE-KFPE (A) and the product of enzymatic reaction after incubated with DPP-4 (B)

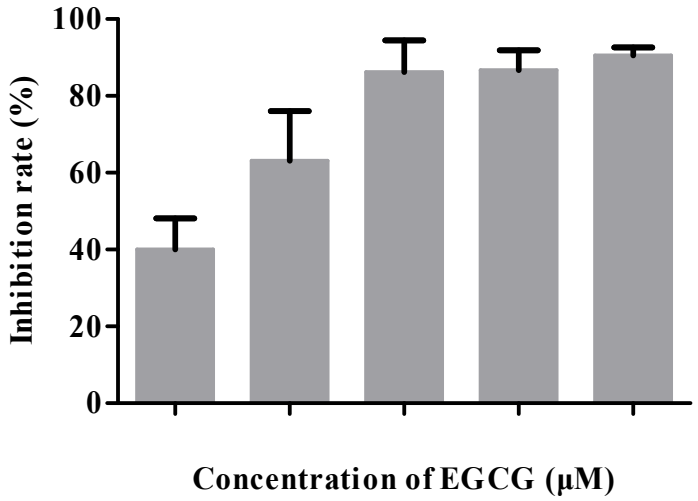


Figure S8 Dose-dependent inhibition of DPP-4 by EGCG

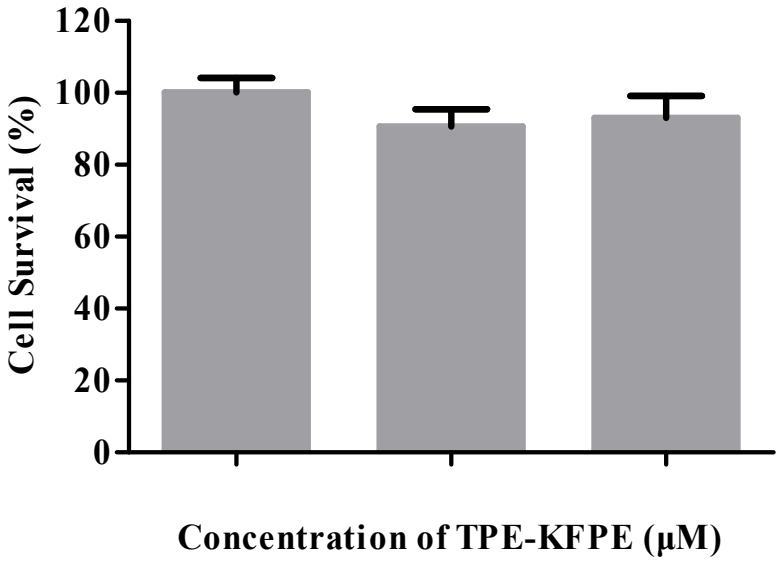


Figure S9 Cytotoxicity of TPE-KFPE on 3T3-L1 measured by MTT assay

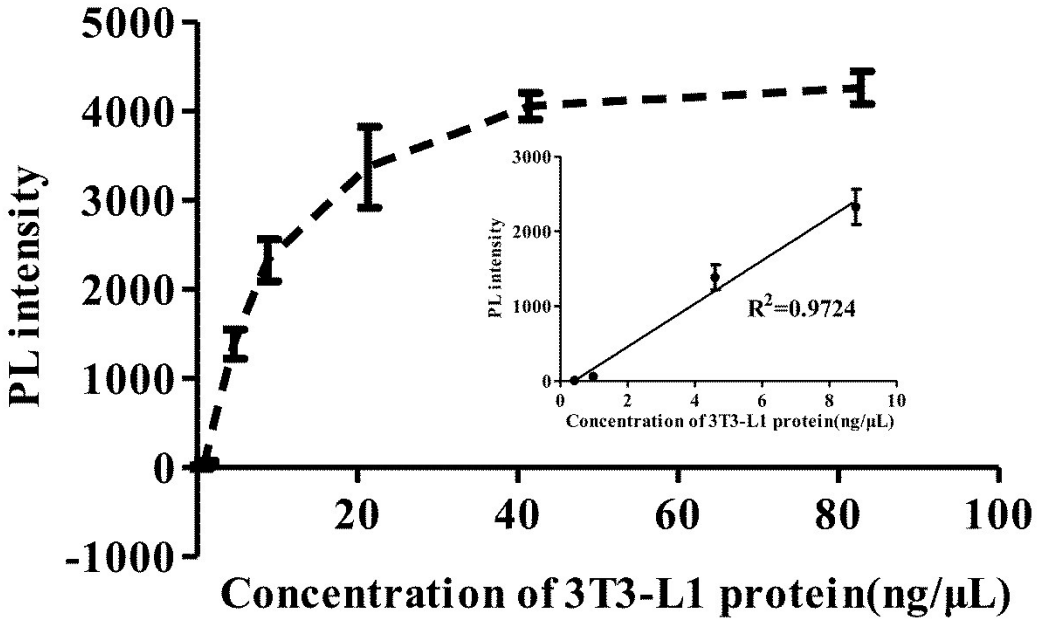


Figure S10 Dose-dependent relationship of DPP-4 concentration in 3T3-L1 cells and fluorescent signal measured by microplate reader

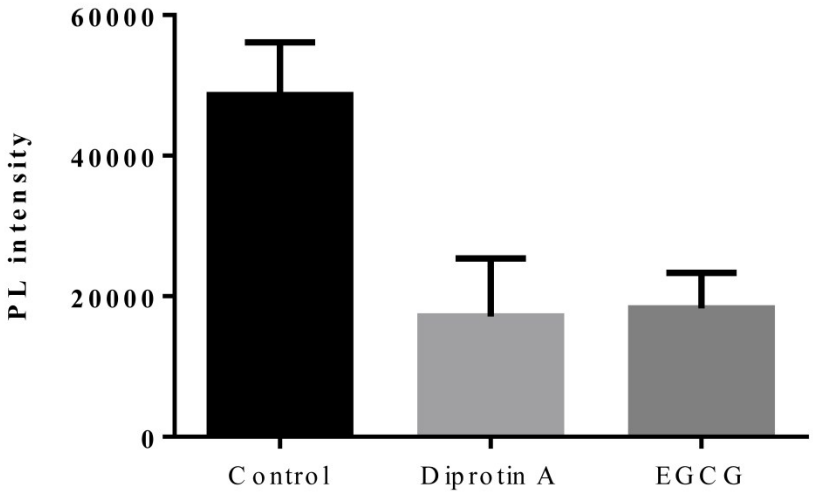


Figure S11 Fluorescent intensity of DPP-4 in 3T3-L1 cells and 3T3-L1 cells treated by diprotin A and EGCG

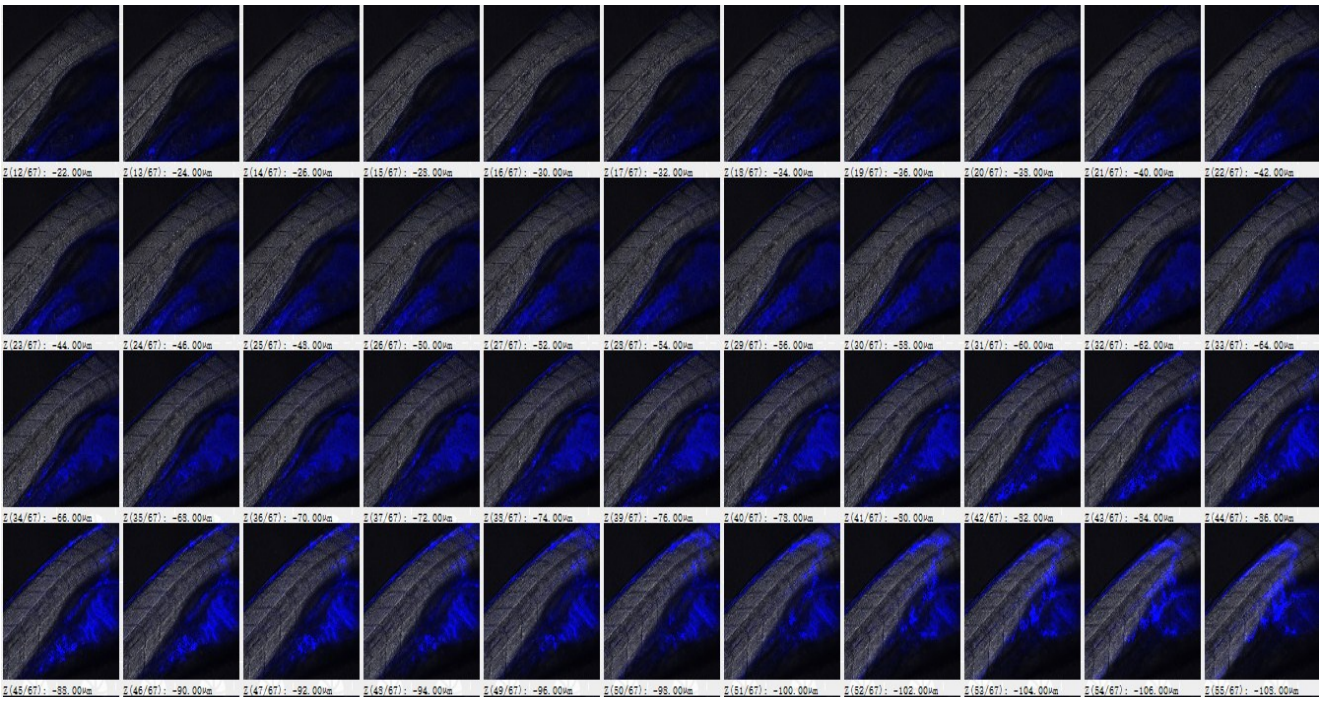


Figure S12 Representative series of optical sections captured by laser scanning confocal along z-axis from the bottom to the top of the islet at 2-μm increments

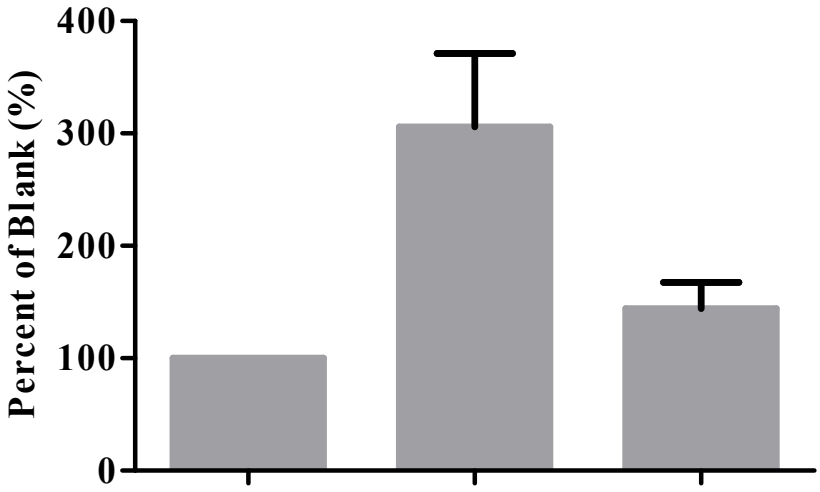


Figure S13 Relative fluorescent intensity of DPP-4 in zebrafish and zebrafish treated by diprotin A