Supplementary Information for

A novel aggregation-induced emission based fluorescent probe for angiotensin converting enzyme (ACE) assay and inhibitor screening

Haibo Wang[a], Yi Huang[a], Xiaoping Zhao [b], Wan Gong [b], Yi Wang [a*], and Yiyu Cheng[a]

[a]College of Pharmaceutical Sciences, Zhejiang University (Zijingang Campus), Hangzhou, 310058, P. R. China.

[b]College of Preclinical Medicine, Zhejiang Chinese Medical University, Hangzhou 310053, PR China.

Experimental details

Materials

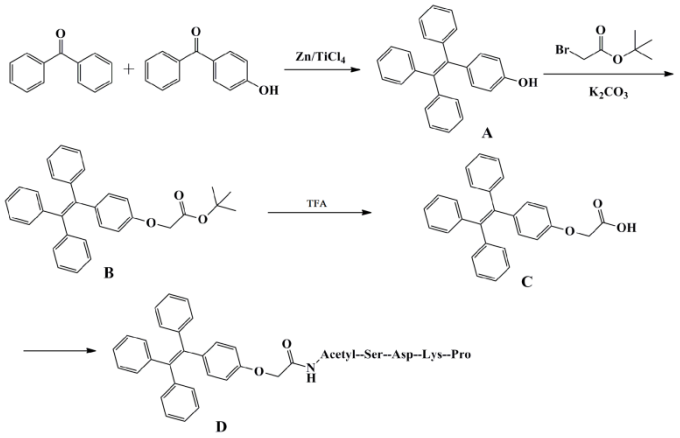
Angiotensin converting enzyme (ACE), bovine serum albumin (BSA) and Cytochrome c (CYC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Solarbio (Beijing, China). Collagenase type I and II were purchased from Gibco (Grand Island, NY, USA). Albumin from human (HSA) was purchased from TCI (Tokyo, Japan). M199 medium and Fetal Bovine Serum (FBS) were purchased from Corning Incorporated Life Sciences (Tewksbury, MA, USA).Endothelial cell growth supplement (ECGS) and epidermal growth factor (EGF) were purchased from BD Biosciences (Bedford, MA, USA).Zinc powder, benzophenone, 4-Hydroxybenzophenone, $TiCl_4$, tert-Butyl bromoacetate, K_2CO_3 were purchased from Aladdin. Methylene dichloride was dried by CaH_2 and THF was refluxed with Na wire before use. Other reagents without being listed were used as received.

Synthesis of TPE-Ser-Asp-Lys-Pro(TPE-SDKP)

In order to synthesize TPE-SDKP, the first step is to synthesize TPE-COOH. Under an N_2 atmosphere, benzophenone(18.2g, 0.1 mol), 4-Hydroxybenzophenone (19g, 0.1mol),zinc powder (16 g, 0.24mmol) and 400 mL THF were added into a three-necked flask equipped with a magnetic stirrer at 0 °C. Then,$TiCl_4$ (13 mL, 0.12mmol) was slowly added with the temperature under 10 °C. The mixture stirred at the room temperature for 0.5 h, then reflux overnight. After the mixture cooled to room temperature, 50 mL dilute hydrochloric acid (1 mmol L^{-1}) was added and extracted with DCM. The crude product was purified by a silica gel column and a white solid was obtained (A).tert-Butylbromoacetate (2 g, 10mmol), A (3.5 g, 10 mmol), K_2CO_3 (2 g, 15 mmol) and 50 mL acetonitrile were added into a flask. The mixture was reflux overnight at 100 °C. The resulting mixture solution was separated by filtration. The crude product was purified through silica gel column. This produce was added

into a solution (DCM:TFA = 1:1) and stirred vigorously. After 3 h, the mixture solution was pour into water and extracted with DCM for three times. The collected organic layer was concentrated under reduced pressure. The obtained white solid was TPE-COOH.^{1}H NMR (500 MHz, $CDCl_3$): 7.12-7.16 (m, 9H), 7.0–7.1 (m, 6H), 6.99 (d, 2H), 6.69 (d, 2H), 4.63 (s, 2H).ESI-MS=405.29

TPE-SDKP was synthesized through solid phase peptide synthesis. ESI-MS=834.79. Purity: 98%.



Scheme S1 Detailed synthetic route of TPE-Ser-Asp-Lys-Pro.

TPE-SDKP fluorescence detection

To verify our previous hypothesis of the quencher peptide of SDKP, which quenched the strong fluorescence of the TPE,TPE-COOH and TPE-SDKP, diluted in distilled water, were prepared with 50μM respectively. The PL spectra, shown as Fig.2A, were measured in the range from 400nm to 600nm withFluoromax-4 spectrofluorimeter (HORIBA JobinYvon, USA).Excitation wavelength was set at 320nm, respectively.

Enzymatic fluorescence and Captopril inhibition

To further validate the enzymatic reaction of ACE hydrolyzing the probe, ACE (18.75mU/mL) was incubated with TPE-SDKP (50μM) in the absence of captopril. Captopril (Sigma, 10nM) was incubated with TPE-SDKP (50μM) and ACE (18.75mU/mL) for 1.5 hours at 37℃, and then $ZnCl_2$ (final concentration 3mM) was added into the system to continue incubating for 0.5 hour at 37℃. The PL spectra were measured at in the range from 400nm to 600nm (Excitation wavelength 320nm) on a JASCO FP-6500 spectrophotometer (JASCO, Tokyo, Japan).

Dose-dependent TPE-SDKP and enzyme

To choose appropriate concentrations of the substrate and enzyme in the assay, different concentrations of TPE-SDKP and ACE were used in the experiment.

Different concentrations of TPE-SDKP ranged from 1 to 100μMwere incubated with ACE (18.75mU/mL) for 1.5 hours at 37℃, respectively. And then $ZnCl_2$ (final

concentration 3mM) was added into the system to continue incubating for 0.5 hour at 37℃. With the increasing concentration of the TPE-SDKP, the PL intensity was gradually increased as the increasing fluorescent hydrolysate TPE-SD was released. In comparison with background, in the absence of substrate, the PL intensity strongly increased.

Different concentration of ACE ranged from 0.625mU to 18.75mU were incubated with TPE-SDKP (50μM) for 1.5 hours at 37℃, respectively. And then $ZnCl_2$ (final concentration 3mM) was added into the system to continue incubating for 0.5 hours at 37℃. With the increasing concentration of ACE, the PL intensity was gradually increased as more enzymes were joined into enzymatic reaction and more fluorescent hydrolysate TPE-SD was released. The PL spectra were measured in the range from 400nm to 600nm (Excitation wavelength 320nm) on JASCO FP-6500.

The study of Enzyme kinetics

To explore the characters of our fluorescent substrate, a typical enzymatic kinetics of ACE has been performed. TPE-SDKP (50μM)and $ZnCl_2$ (3mM) were incubated in the presence or absence of ACE(18.75mU/mL) at 37℃. PL intensity was immediately measured at the interval of one minute and lasted for 3 hours. Fluorescent detection on SpectraMax M2 fluorescence plate reader (Molecular Devices, CA, USA): excitation wavelength 320nm, emission wavelength 470nm. In the presence of ACE, the PL intensity was increasing by time, while the PL intensity sustained a low and stable fluorescence in 3 hours in the absence of ACE.

Dose-dependent enzyme essay

Previous study has presented the general character of dose-dependent ACE reaction, to quantitatively and search the linear range of the ACE, different concentrations of ACE ranged from 0 to 6.25 mU/ml were incubated with TPE-SDKP(200μM) for 2 hours at 37℃, and then $ZnCl_2$ (final concentration 3mM) was added to continue incubating for another hour. The PL intensity was measured in the condition of excitation wavelength 320nm and emission wavelength 470nm on SpectraMax M2 fluorescence plate reader. As shown in Fig.3(B), with the increasing concentration of ACE, the PL intensity was gradually increased.

Dose-dependent inhibition of ACE by captopril

We investigated the dose-dependent ACE inhibitory activity of captopril using TPE-SDKP as the substrate.ACE (18.75mU/mL) was incubated with TPE-SDKP (50μM) in the presence or absence of captopril for 2 hat 37℃, and then $ZnCl_2$ (final concentration 3mM) was added to continue incubating for another hour. The

concentrations of captopril were ranged from 0.5 to 50 nM. The PL intensity was measured in the condition of excitation wavelength 320nm and emission wavelength 470nm on SpectraMax M2 fluorescence plate reader.

Enzymatic specificity

The enzymes are varied in cells membrane and plasma. To explore the specification enzymatic reaction of the probe with ACE (0.25U/ml in working solution), different enzymes/protein was performed in the study. BSA, trypsin, collagenase type I (Coll I)and type II (Coll II), cytochrome C(CYC), and human serum albumin (HSA)were chosen as the comparison samples. Different enzyme/protein samples (0.5mg/mL in working solution)were incubated with TPE-SDKP (50μM) for 2 h at 37℃, and then $ZnCl_2$ (final concentration 3mM) was added into the system to continue incubating for another hour. The PL intensity was measured in the condition of excitation wavelength 320nm and emission wavelength 470nm on SpectraMax M2 fluorescence plate reader.

Identification of active components with ACE inhibitory activity from TMYX

TongmaiYangxin Pills (TMYX), a botanical drug for treating cardiovascular disease, was obtained from LeRenTang pharmaceutical factory (Tianjin, China). Components of TMYX were prepared by preparative chromatography in our lab. Two components, i.e. fraction 20 and fraction 24 with different concentration, were incubated with TPE-SDKP (50μM) and ACE enzyme (12.5mU/mL) for 2h at 37℃,and then $ZnCl_2$ (final concentration 3mM) was added to incubate for another hour. The PL intensity was measured in the condition of excitation wavelength 320nm and emission wavelength 470nm on SpectraMax M2 fluorescence plate reader. The dose dependent efficacy of active component was presented in Fig.S6.The IC_{50} of components calculated by GraphPad Prism was 7.12 and 25μg/mL, respectively.

Cell Culture

The human mesenchymal stem cells (hMSC) and human umbilical vein endothelial cell line (HUVEC) were gifts from Affiliated Hospital of Zhejiang University. HT-29 colon cancer cells (from Cellbank of Chinese Science Academy, Shanghai, China) were cultured in DMEM (Corning) containing 10% fetal bovine serum (Corning), 100U/mL penicillin, and 100 μ g/mL streptomycin. hMSC were cultured in Human Mesenchymal Stem Cell Growth Medium (Cyagen, CA,USA). HUVEC cell line was grown in M199 media with 10% fetal bovine serum containing endothelial cell growth supplement (ECGS) and epidermal growth factor (EGF). The cells were cultured in 5% humidified CO_2 atmosphere at 37 °C.

ACE expression in different cells using TPE-SDKP

Most species of cells barely express ACE in the cells, but some specific species like hMSC and HUVEC could express ACE. We employed HT-29, hMSC and HUVEC

into exploring whether the probe could be an indication tool for ACE expression in different cells. After each sample cells were seeded into dishes and incubated for 24 h (HUVEC incubated for 48 h), TPE-SDKP (50μM，diluted in D-hanks，50mM Tris added) was added into each samples to incubate for 2 h in 5% humidified CO_2 atmosphere at 37 °C, and then $ZnCl_2$ (final concentration 3mM) was added to continue incubating for another hour. Fluorescent images were captured by ZEISS AXIO Observer.A1 fluorescence microscope with a DAPI filter.HT-29 and hMSC were captured with 40x lens and HUVEC was captured with 100x microscope lens. As shown in Fig.S7, HT-29 present a barely visible fluorescent signal with the probe, while hMSC and HUVEC exhibited a relatively strong signal, which is in agreement with previous reported literature.

Imaging of stimulated HUVEC using TPE-SDKP

After seeded into plates and 24 hours cultivation, the cell line HUVEC was treated with phorbalmyristate acetate (PMA, 250nM) for up-regulation group; meanwhile, inhibition group was treated with PMA and captopril (Cap, 100nM). After 36 hours incubation of HUVEC, the medium was discarded and the cells were washed with D-Hanks. TPE-SDKP was diluted with D-Hanks (50μM，50mM Tris added，pH7.5) and was added immediately into cells to incubate for 2 h in 5% humidified CO_2 atmosphere at 37 °C, and then $ZnCl_2$ (final concentration 3mM) was added to continue incubating for another hour. Fluorescent images were captured by ZEISS AXIO Observer.A1 fluorescence microscope with a DAPI filter. Fig.3 present the typical images of each samples, we then summed the fluorescent intensity with the software ImageJ, relative folds compared with control (normal) cells were present as Fig.S8. About 3.7 folds of enzymatically activity ACE were observed by using the probe compared with normal cells, and the fluorescent intensity was decreased sharply by adding the ACE inhibitor captopril.

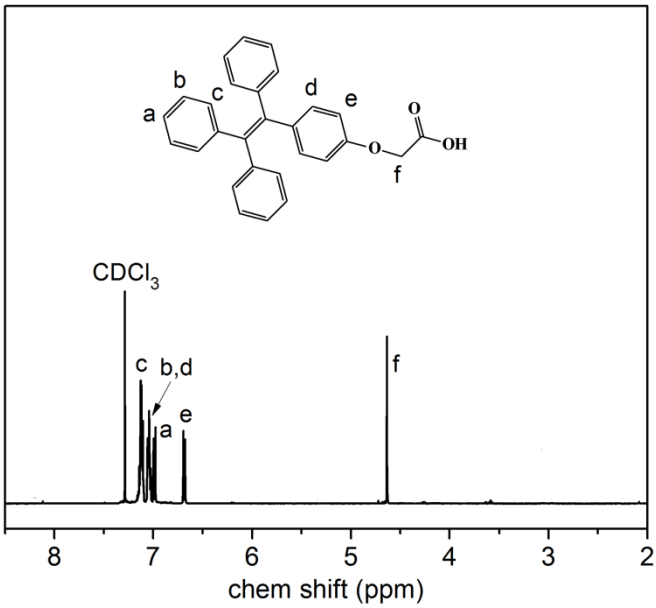


Fig.S1[1]H NMR spectrum of TPE-COOH ($CDCl_3$).

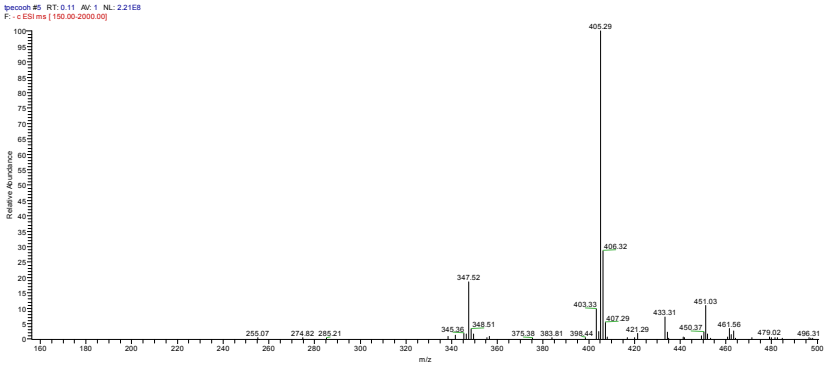


Fig.S2 Mass spectrum of TPE-COOH.

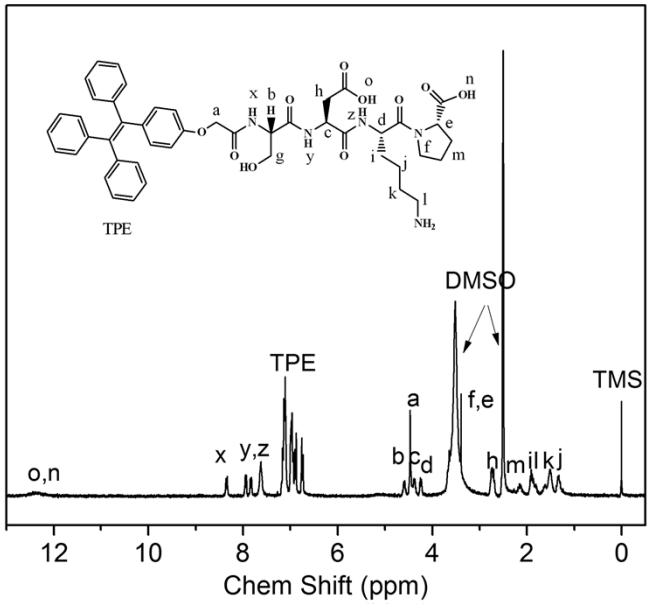


Fig.S3 [1]H NMR spectrum of TPE-SDKP (DMSO).

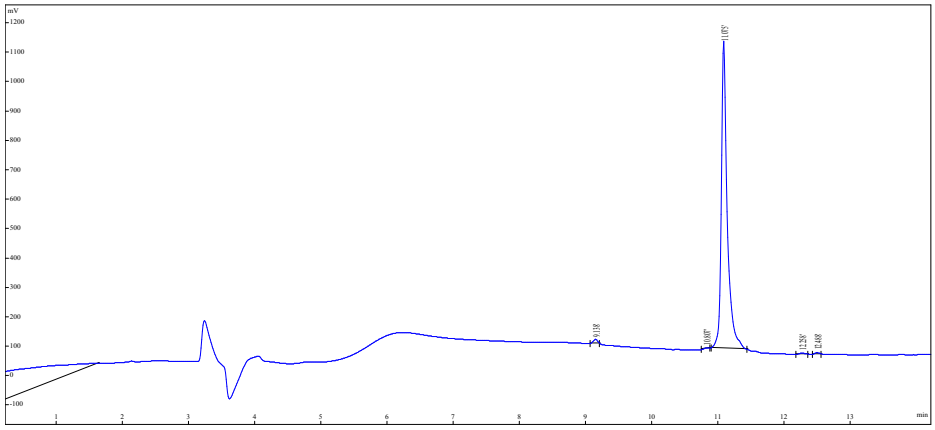


Fig.S4 HPLC chromatogram ofTPE-SDKP

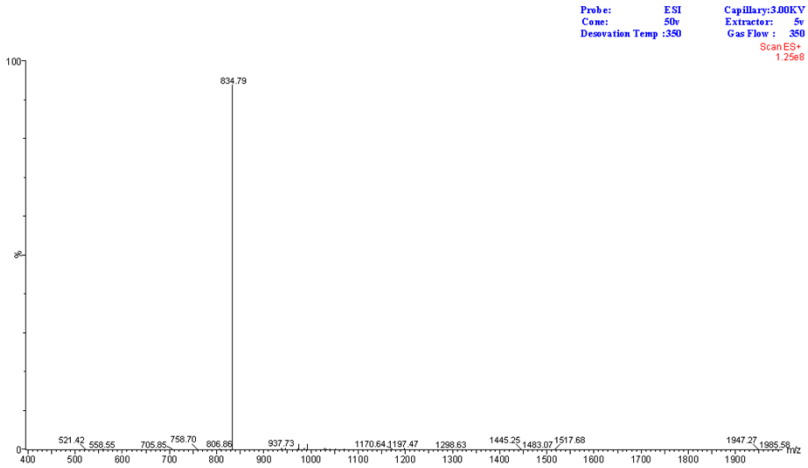


Fig.S5 Mass spectrum of TPE-SDKP.

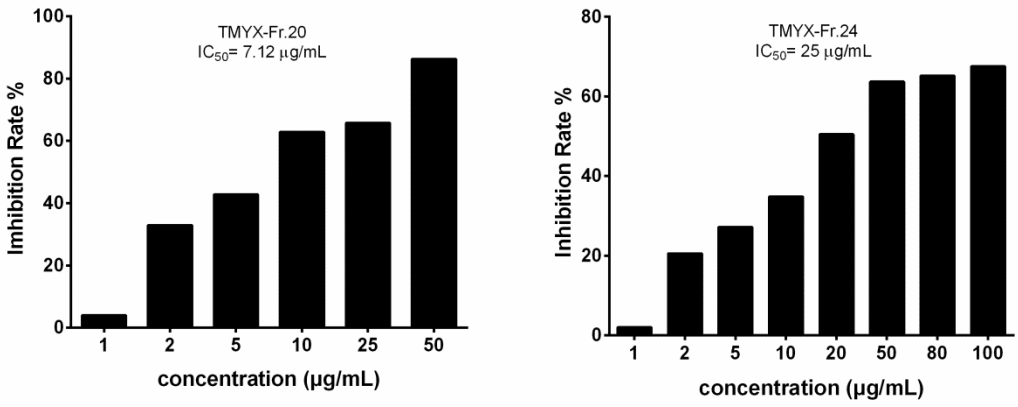


Fig.S6 Dose dependent inhibition of two active components from TMYX on ACE activity

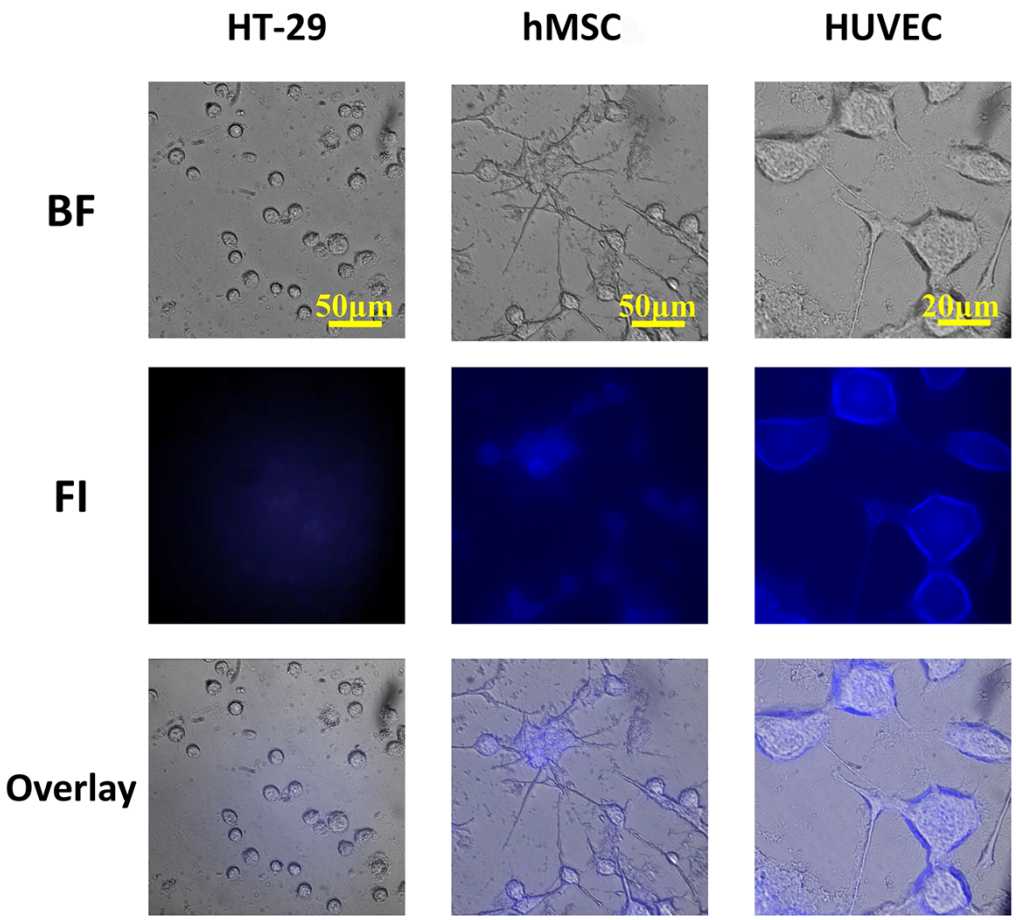


Fig.S7 Bright-field (BF), fluorescence (FL), and overlay images of HT29, HUVEC, and hMSC cells. The images were acquired using a fluorescence microscope (ZEISS) equipped with DAPI filter.

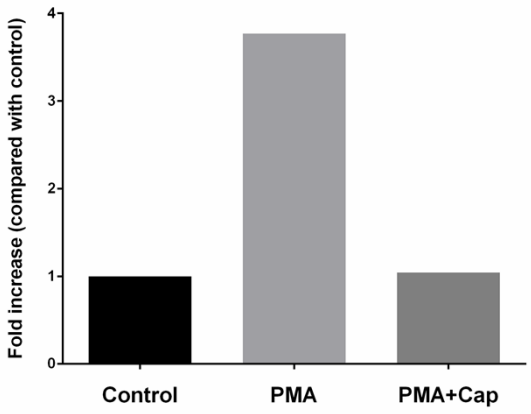


Fig.S8 Relative fluorescence intensity of HUVEC cells listed by normal, treated by PMA, and treated with PMA plus captopril.