Specific turn-on fluorescent probe with aggregation-induced emission characteristics for SIRT1 modulator screening and living-cell imaging

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Supplementary Information

Experimental details

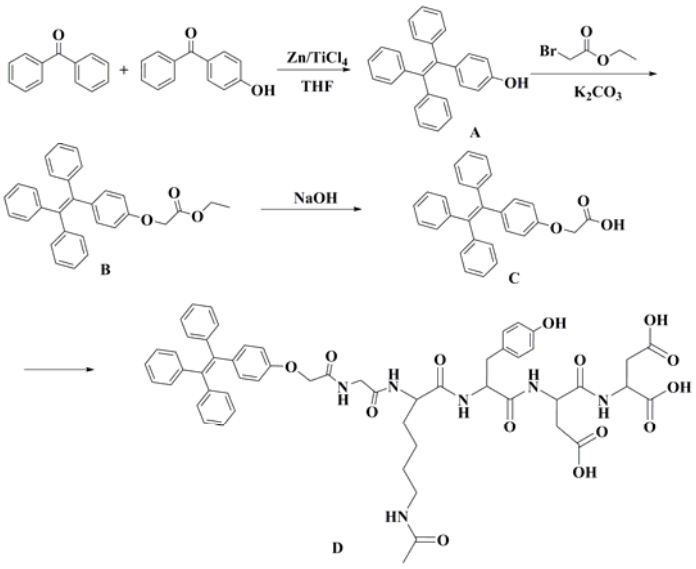
Materials

SIRT1 (human recombinant), lysyl endopeptidase cut and HDAC1 were purchased from Cayman Chemical (USA). Trypsin was purchased from Solarbio (China). HDAC3 was purchase from Sino Biological Inc (China). Collagenase type I, II, DMEM/F-12 medium and fetal bovine serum (FBS) were purchased from Gibco (USA). Cytochrome C (CYC), bovine serum albumin (BSA), thrombin, resveratrol, EX527 and lysozyme were purchased from Sigma-Aldrich (USA). SRT1720 was purchased from Apexbio (USA). Human serum albumin was purchased from TCI (Japan). DMEM medium was purchased from Corning Incorporated Life Sciences (USA). BCA protein assay kit and ECL western blotting substrates were purchased from Thermo (USA). SirT1 antibody was purchased from cell signaling technology (USA). Actin antibody, and Goat anti-Rabbit/anti-mouse IgG/HRP were purchased from Beyotime (China). Benzophenone, 4-Hydroxybenzophenone, $TiCl_4$, K_2CO_3 were purchased from Aladdin. Gly-Lys(Ac)-Tyr-Asp-Asp was synthesized by Shanghai Top-peptide Bio (China). Other reagents without being listed were used as received.

Synthesis of TPE-Gly-Lys(Ac)-Tyr-Asp-Asp (TPE-GK(Ac)YDD)

The first step of synthesize TPE-GK(Ac)YDD is to prepare TPE-COOH. Under an N_2 atmosphere, 0.12 mol of benzophenone, 0.1 mol of 4-Hydroxybenzophenone and 0.44 mol of zinc powder were added into a three-necked flask equipped with a magnetic stirrer. The flask was degassed under vacuum and flushed with nitrogen three times and 400 mL newly dried THF was added. Then, 0.22 mol of $TiCl_4$ was slowly added with the temperature less than 10 °C. The mixture stirred at 85 °C. After the mixture cooled to room temperature, 80 mL dilute hydrochloric acid (1 $mmol \cdot L^{-1}$) was added and extracted with DCM. The crude product was purified by a silica gel column and a white solid was obtained Compound A. 10 mmol of ethyl bromoacetate, 10 mmol of A, 15 mmol of K_2CO_3 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 to get Compound B. This produce was added into a solution (THF:H_2O = 7:3) with equivalent of NaOH and stirred vigorously. After 24 hours, 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 (Compound C). Through solid phase peptide synthesis, amino group of specific designed peptide was reacted to TPE-COOH to obtain TPE-GK(Ac)YDD (Compound D). ESI-MS=1026.6. Purity: 96.6% was obtained by HPLC. The ^{1}H NMR spectrum of TPE-GK(Ac)YDD (DMSO) was shown in Fig.S1, while the mass spectrum and HPLC chromatogram were shown as Fig.S2 and Fig.S3.



Scheme S1 Detailed synthetic route of TPE-GK(Ac)YDD.

TPE-GK(Ac)YDD fluorescence detection

The PL spectra of TPE-COOH and TPE-GK(Ac)YDD (50 μM diluted in Tris-HCl, pH 8.8), were measured from 400nm to 600nm with JASCO FP-6500 spectrophotometer (JASCO, Japan), while excitation wavelength was fixed at 320nm, shown as Fig.S4.

Enzymatic fluorescence and moderators

In order to ensure the deacetylation reaction of SIRT1, SIRT1 (60 μg/mL) was added to TPE-GK(Ac)YDD (20 μM) and NAD^+ (Sigma, 3 mM) for 3 hours incubation at 37℃ in the presence or absence of the inhibitor EX527 (200 nM), activator SRT1720 (500 nM) and lysyl endopeptidase (0.7 μg/mL). The PL spectra were measured from 400nm to 600nm (Excitation wavelength 320nm) with JASCO FP-6500 spectrophotometer. AIE can be produced by deacetylated products TPE-GKYDD, but the fluorescence can be amplified by lysyl endopeptidase (shown in Fig.S6 and Fig.1A).

Optimal pH of Tris-HCl buffer

To explore the optimal pH of Tris-HCl buffer, TPE-GK(Ac)YDD (20 μM) was incubated with NAD^+ (3 mM), lysyl endopeptidase (1.4 μg/mL) and SIRT1 (57.6 μg/mL) for 3 hours inbucation at 37℃ in pH 6.5, 7.5, 8.8 Tris-HCl buffer, respectively. Fluorescence intensity was recorded by TECAN infinite F200 Multi-function microplate with the following parameters: excitation wavelength 320 nm, emission wavelength 465 nm. As shown in Fig.S7, the difference was compared by calculated $(I-I_0)/I_0$, I_0 represented the PL intensity in the absence of any enzyme except lysyl endopeptidase, while I represented the PL intensity in the presence of SIRT1.

Dose-dependent manners of TPE-GK(Ac)YDD and SIRT1

To obtain the optimal concentration of the probe and enzyme, TPE-GK(Ac)YDD and SIRT1 were incubated with varied concentration. This assay was carried out at 37℃ for 3 hours, while using different concentrations of TPE-GK(Ac)YDD (1, 5, 10, 15, 20, 25 and 30 μM). The concentration of NAD^+, SIRT1 and lysyl endopeptidase were 3 mM, 80 μg/mL and 0.7 μg/mL, respectively. The PL intensity was gradually increased along with the increasing concentration of the probe, due to the increasing generation and aggregation of hydrolyzed products TPE-G. Also, there is big difference between the PL intensity of background and hydrolyzed products.

The assay of dose-dependent SIRT1 was also carried out at 37℃ for 3 hours, while using different concentrations of SIRT1 (4, 10, 20, 30, 40, 50, 60, 80 μg/mL). The concentration of NAD^+ and lysyl endopeptidase were the same as above, while the concentration of TPE-GK(Ac)YDD was 20 μM. The PL intensity was gradually increased along with the increasing concentration of SIRT1, due to the increasing generation and aggregation of hydrolyzed products TPE-G with participation of more SIRT1 in the reaction. The PL spectra were measured from 400nm to 600nm (Excitation wavelength 320 nm) with JASCO FP-6500 spectrophotometer.

Enzyme kinetics assay

With or without SIRT1 (60 μg/mL), NAD^+ (3 mM) and lysyl endopeptidase (1.4 μg/mL) were add to TPE-GK(Ac)YDD (20 μM) for incubation at 37℃. Fluorescence intensity was measured by TECAN infinite F200 Multi-function microplate (Tecan, Austria) with the parameters: excitation wavelength 320 nm, emission wavelength 465 nm. The PL intensity was recorded at the interval of 80 seconds until PL intensity was approximately stable.

Linear range of the assay detecting SIRT1

To explore the approximate linear range of SIRT1, different concentrations of SIRT1 ranged from 0.5, 1, 2, 4, 10, 20, 30, 40, 50, 80, 100, 120, 150, 200 μg/mL were added into the mixture of NAD^+ (3 mM), lysyl endopeptidase (1.4 μg/mL) and TPE-GK(Ac)YDD (20 μM) for 2.5 hours incubation at 37℃. Fluorescence intensity was recorded by TECAN infinite F200 Multi-function microplate with the same parameters as enzyme kinetics assay. Along with the increasing concentration of SIRT1, the PL intensity was increased (shown in Fig.2B).

Dose-related inhibition of SIRT1 by EX527

To explore the dose-related effects of EX527, SIRT1 was incubated with different concentration of EX527 ranged from 0, 10, 25, 50, 100, 200, 500, 750, 1000, 2000 nM for 3 hours at 37℃. Reaction system was the same as enzyme kinetic assay.

Fluorescence intensity was recorded by TECAN infinite F200 Multi-function microplate with the same parameters as enzyme kinetics assay.

Dose-related activation of SIRT1 by SRT1720

To explore the dose-related effects of SRT1720, SIRT1 was incubated with different concentration of SRT1720 ranged from 10, 25, 50, 200, 500, 1000 nM for 3 hours at 37℃. Reaction system was the same as enzyme kinetic assay. Fluorescence intensity was recorded by TECAN infinite F200 Multi-function microplate with the same parameters as enzyme kinetics assay. As shown in Fig.S8, SRT1720 exert its activation effect to SIRT1 in good dose-depend manner.

The study of enzymatic specificity

In order to exclude the possibility of unspecific reactions between probe and other histone deacetylases (HDACs) or proteins existed in cell culture medium., SIRT1, human serum albumin (HSA), bovine serum albumin (BSA), collagenase type I (Coll I), type II (Coll II), cytochrome C (CYC), lysozyme and trypsin at the concentration of 60 μg/mL, thrombin (0.2 U/mL) were added to the mixture of NAD^+ (3 mM), lysyl endopeptidase (1.4 μg/mL) and TPE-GK(Ac)YDD (20 μM) for 3 hours inbucation at 37℃. Moreover, histone deacetylases, HDAC1 and HDAC3 (50 μg/mL) was also used. Fluorescence intensity was recorded by TECAN infinite F200 Multi-function microplate with the same parameters as enzyme kinetics assay. The difference was compared by calculated $(I-I_0)/I_0$ (shown in Fig.2D), it is obvious that TPE-GK(Ac)YDD is the specific substrates for SIRT1.

LC-MS Analysis

Samples analyzed by Finnigan LCQ Deca XP^{plus} ion trap mass spectrometer for MS (Thermo Finnigan, USA). The acquisition parameters for LC/ESI-MS were as follows: collosion gas, high-purity helium (He); nebulizing gas, high purity nitrogen (N_2); ion spray voltage: −3 kV; capillary temperature: 350 ℃; capillary voltage: −15 V; tube lens offset voltage: −30 V; mass range：*m/z* 100−1500. Chromatographic separation was carried out on a reversed-phase Zorbax SB-C18 analytical column (250 mm × 4.6 mm I.D., 5 μm, Agilent Technologies, USA). The mobile phase

consisted of water containing 0.1% (v/v) formic acid (A) and acetonitrile (B). A gradient program was used according to the following profile: 0 min, 50% B; 5 min, 50% B; 30 min, 95% B; The flow rate was 0.6 mL/min.

Cell Culture

H9c2 (from Cell bank of Chinese Science Academy, Shanghai, China) were cultured in DMEM (Corning, USA) containing 10% fetal bovine serum (Corning, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA). Neonatal cardiomyocytes was separated from myocardial tissue of newborn SD rats. The cells were cultured in DMEM/F-12 (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, USA). 0.1mM BrdU (Sigma, USA) was also added to prevent the proliferation of fiberblasts. All the cells were cultured in 5% humidified CO_2 atmosphere at 37 °C.

Cytotoxic of TPE-GK(Ac)YDD in H9c2 cells

H9c2 cells were seeded into 96-well microplate ($4x10^4$/ml), then treated with TPE-GK(Ac)YDD (50 μM) for 3, 6, 12, 24 hours. CellTiter-Glo® Luminescent Cell Viability Assay was used to test cell activity.

Cell survival (%) = luminescent intensity of test sample/luminescent intensity of control× 100%

Living cell imaging of SIRT1 by fluorescent microscope using TPE-GK(Ac)YDD

The cells were seeded into dishes and incubated. The previous culture medium was replaced by fresh medium. Next, 3 hours incubation with TPE-GK(Ac)YDD (50 μM) in incubator at 37 °C was followed. After the dishes were washed by PBS, fluorescent images were obtained by ZEISS AXIO Observer.A1 fluorescence microscope equipped with a DAPI filter. As shown in Fig.S11, a strong fluorescence signal was exhibited in H9c2 nucleus region.

Image of Neonatal cardiomyocytes treated with SIRT1 activator and inhibitor using TPE-GK(Ac)YDD

After being primary cultured for 48 hours, neonatal cardiomyocytes were exposed to resveratrol (20 μM) and EX527 (20 μM) for 12 hours incubation. The previous culture medium was replaced by fresh medium, followed by 3 hours incubation in incubator with TPE-GK(Ac)YDD (50 μM) at 37 °C. Before fluorescent images were obtained by Nikon A1R laser scanning confocal microscope equipped with 405nm laser, the dishes were washed by PBS. The images were captured with 60x lens. As shown in Fig.4, a weak fluorescent signal with the probe was presented in EX527 treated cell, while a strong fluorescence signal was exhibited in resveratrol treated cell. The cells were also stained with fluorescein diacetate (FDA) (10 μg/mL) for 10 minutes after the incubation with TPE-GK(Ac)YDD (50 μM), the dual fluorescent images of neonatal cardiomyocytes were captured by Nikon Laser scanning confocal microscope with 60x lens. Excitation and emission wavelength: 488 nm and 525 nm for FDA; 405 nm and 450 nm for TPE- GK(Ac)YDD (shown as Fig.3). The fluorescence intensity was calculated with the software Image-J by summing the mean intensity of each cell from six pictures.

Imaging of wild-type rMSC and SIRT1$^{-/-}$ rMSC using TPE-GK(Ac)YDD

Construction of the recombinant lentivirus with SIRT1 was performed by ShangHai SBO Medical Biotechnology Company. Briefly, interference shRNA of SIRT1 is synthesized to complementary oligonucleotide sequences based on the target sequence of 5 '- GCAGATTAGTAAGCGTCTT -3' and unrelated control sequences of 5 '-TTCTCCGAACGTGTCACGT-3', digested by Age I and EcoR I, and linked with pMAGic 7.1-EGFP-Puro carrier to construct lentiviral vector. Then, obtained virus particles after transfecting into 293T cell with the packaging plasmid psPAX2 and pMD2.G. Primary cultured rat mesenchymal stem cells (rMSCs) were kindly provided by Dr. Ling Zhang in The Second Affiliated Hospital Zhejiang University, School of Medicine. For MSCs infection, cells were seeded at a density of 1 × 10^5 cells in a 6-well plate and infected with lentiviral vectors in the presence of 10 μg/mL polybrene (Millipore). At 12hrs post-infection, the growth medium was replaced. 48hrs later, the GFP expression of transduced cells was observed under

fluorescence microscopy and then the transfected cells were cultured in a 5% CO_2-humidified incubator at 37℃.

The fluorescent imaging of WT rMSCs and SIRT1$^{-/-}$ rMSC were also obtained by Nikon A1R laser scanning confocal microscope equipped with 405nm laser. The images were captured with 60x lens. As shown in Fig.S13(A), a weak fluorescent signal with the probe was presented in SIRT1$^{-/-}$ rMSC, while a strong fluorescence signal was exhibited in normal cell. The fluorescence intensity was calculated with the software Image J by summing the mean intensity of each cell from four pictures. The cells lysate were also analyzed by western blot to check the expression of SIRT1 in normal cells and SIRT1$^{-/-}$ rMSC. Briefly, MSC were seeded into dishes. The cells were lysised to obtain the cytoplasmic extracts. The concentration of total protein was measured by BCA assay kit. Protein samples were mixed with loading buffer and reducing agent, then separated by 10% Bris-tris gel, bloted on a PVDF membrane. Subsequently, the membrane was blocked in blocking buffer (5% bovine serum albumin), and incubated with primary antibody of SIRT1 (1:500 in blocking buffer), Actin (1:2000 in blocking buffer) overnight, washed for 3 times and then incubated with HRP anti-rabbit/mouse IgG for 1h at room temperature. ECL chemiluminescence reagent was used to visualize blots. The bands were exposed by Bio-Rad ChemiDoc XRS.

Dose-related activation of SIRT1 by Ophiopogonin D'

We found two compound in primary screening of SIRT1 moderator, ophiopogonin D' and 20(S)-Ginsenoside Rg_3. To explore the dose-related effects of ophiopogonin D', SIRT1 was incubated with TPE-GK(Ac)YDD (20 μM) with different concentration of ophiopogonin D' ranged from 0.1, 0.5, 1, 5, 10, 25 μM for 3 hours at 37℃. Fluorescence intensity was recorded by TECAN infinite F200 Multi-function microplate with the same parameters as enzyme kinetics assay. As shown in Fig.S10, ophiopogonin D' exerted dose-dependent activation on SIRT1 activity.

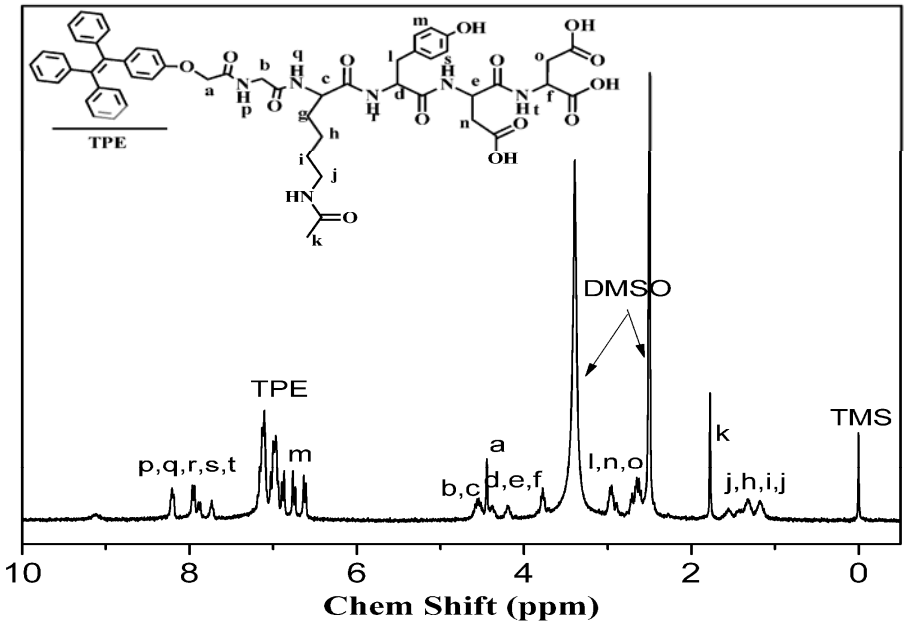


Figure S1 ^{1}H NMR spectrum of TPE-GK(Ac)YDD (DMSO).

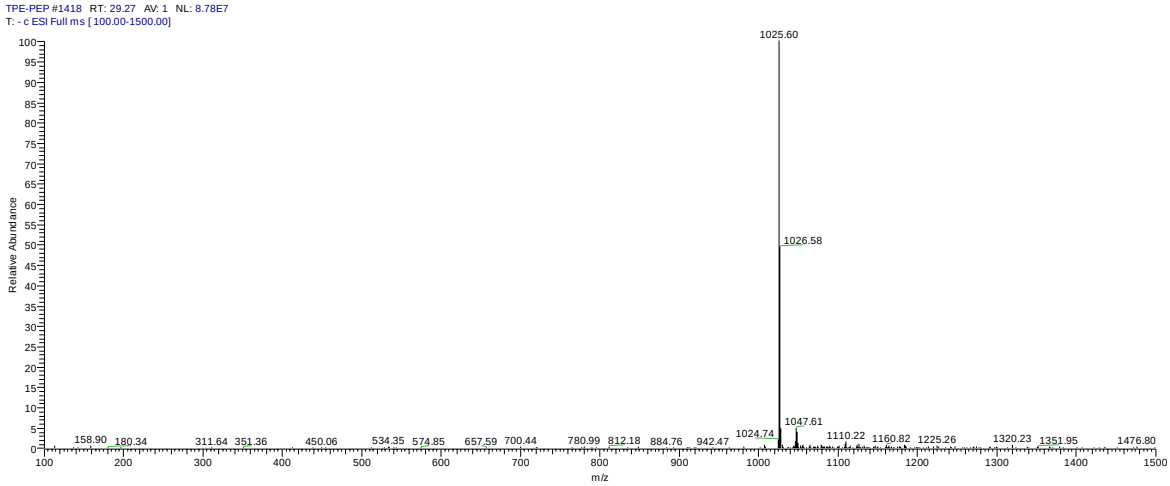


Figure S2 Mass spectrum of TPE-GK(Ac)YDD.

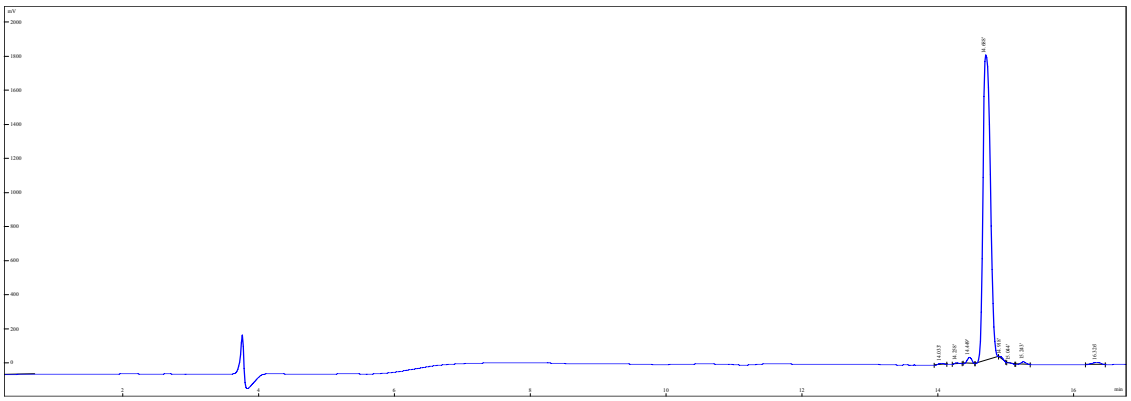


Figure S3 HPLC chromatogram of TPE-GK(Ac)YDD

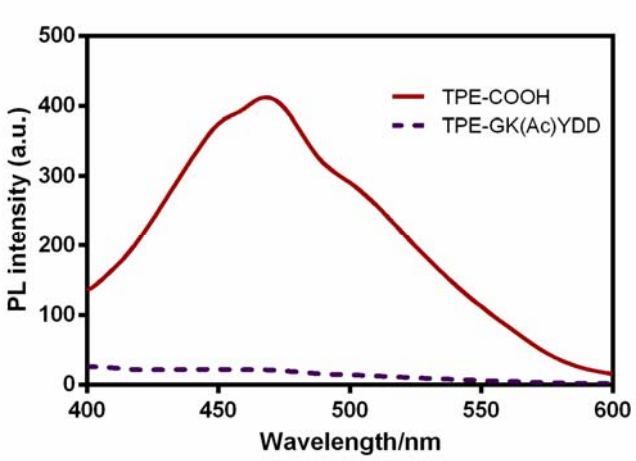


Figure S4 PL spectra of the TPE-COOH and TPE-GK(Ac)YDD

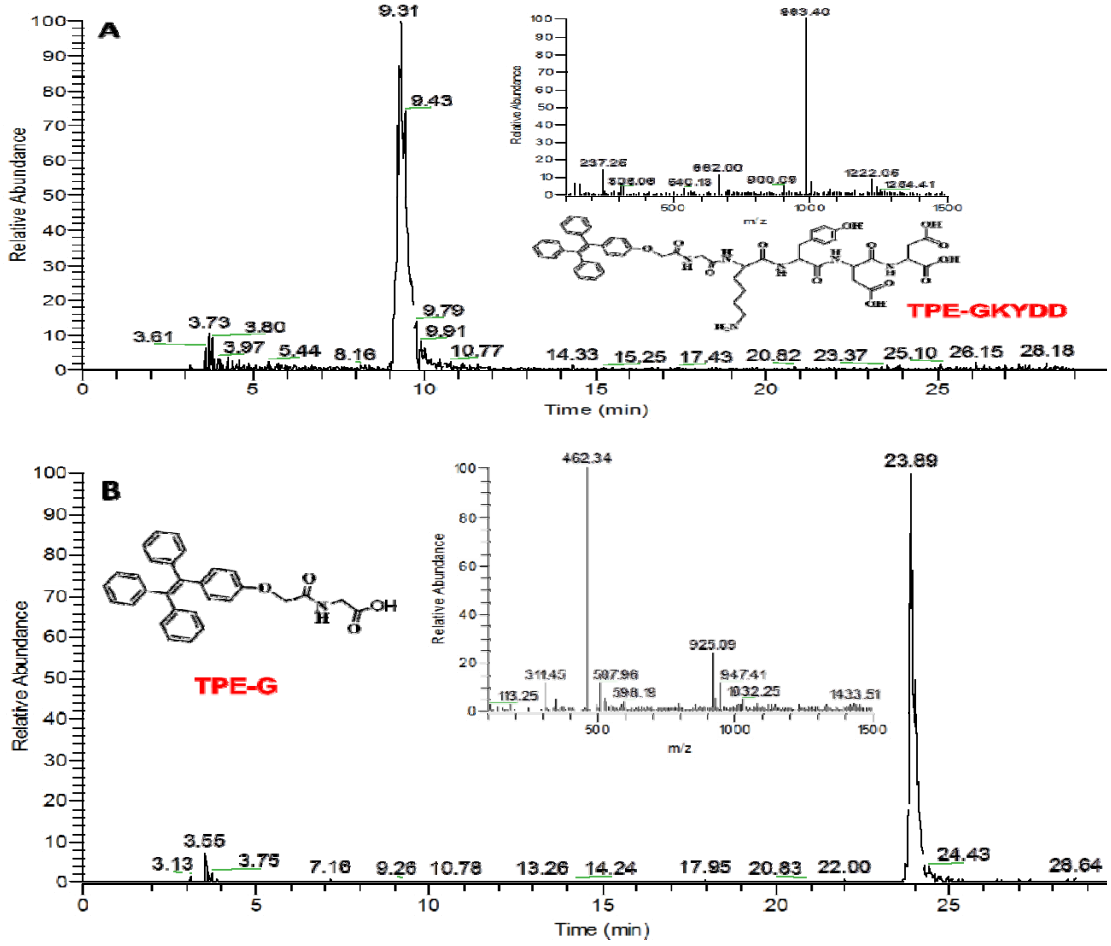


Figure S5 LC-MS chromatogram and structure deacetylated products of TPE-GK(Ac)YDD incubation with SIRT1. (A) TPE-GKYDD was identified in assay system without lysyl endsopeptidase (B) TPE-G was identified in assay system with lysyl endopeptidase

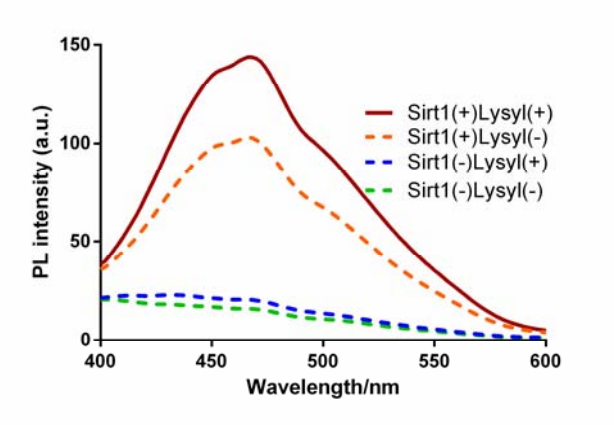


Figure S6 PL spectra of the probe in the presence or absence of SIRT1 and lysyl endopeptidase

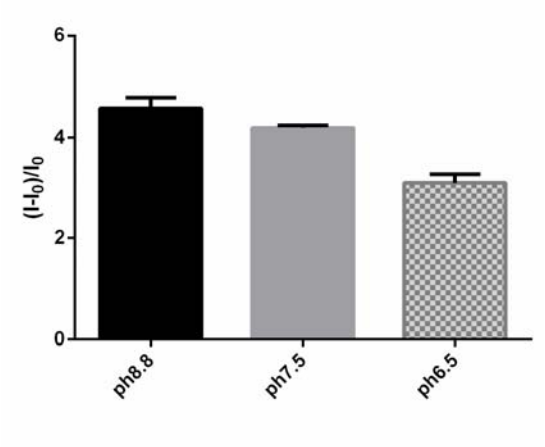


Figure S7 The calculated $(I-I_0)/I_0$ value of SIRT1 when the enzyme reaction was carried out in the Tris-HCl buffer with different pH value.

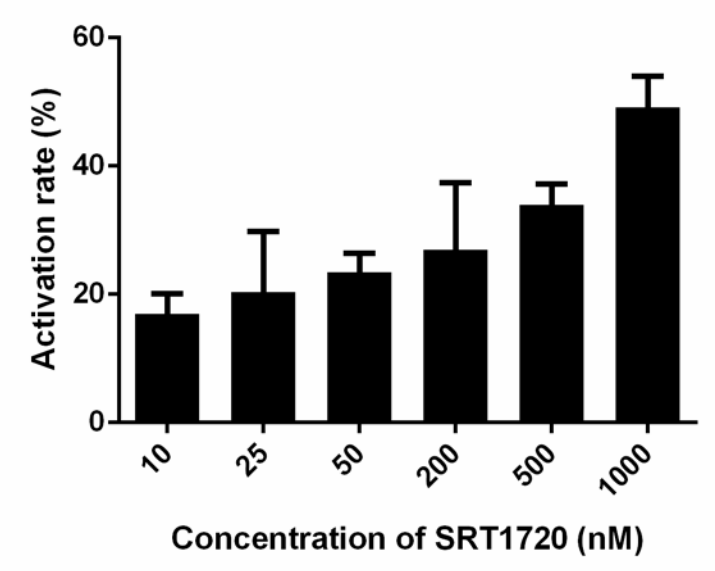


Figure S8 The dose-dependent activation of SRT1720 on SIRT1 activity.

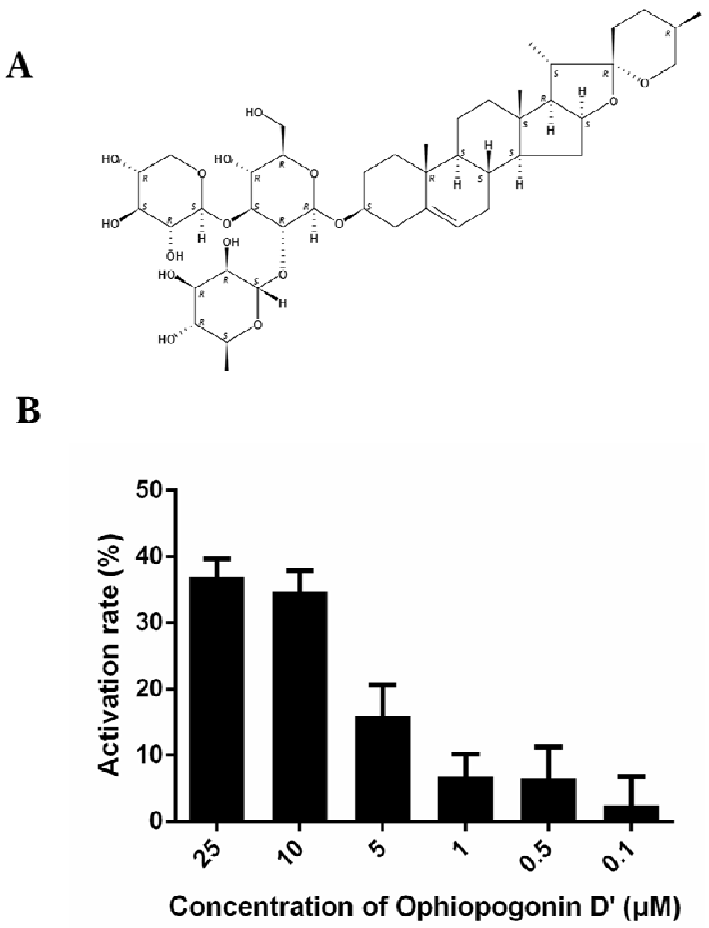


Figure S9 (A) The structure of ophiopogonin D'

(B) The dose-dependent activation of ophiopogonin D' on SIRT1 activity.

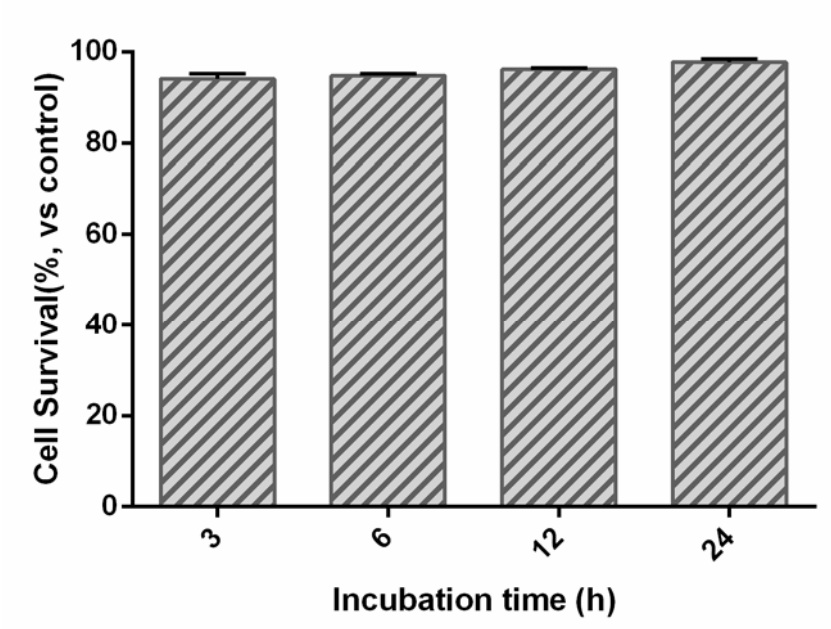


Figure S10 Cytotoxicity of TPE-GK(Ac)YDD (50 μM) with CTG assay

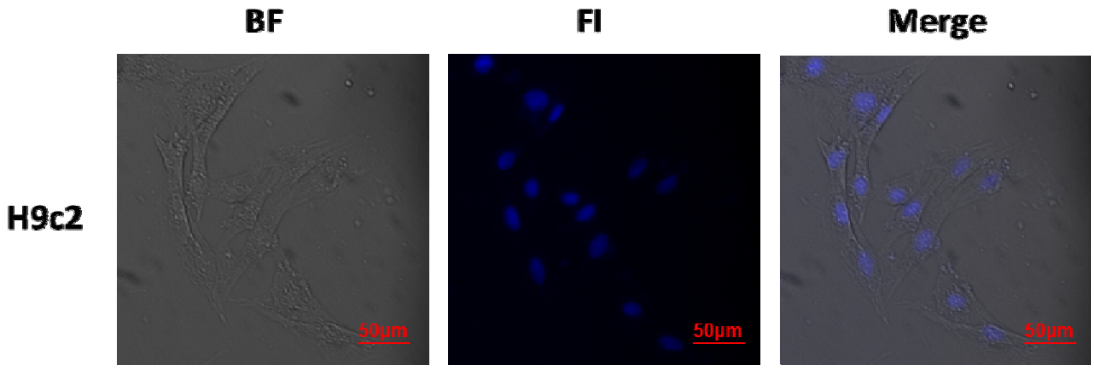


Figure S11 Bight-field (BF), fluorescence (FL), and overlay images of H9c2 cardiomyocytes

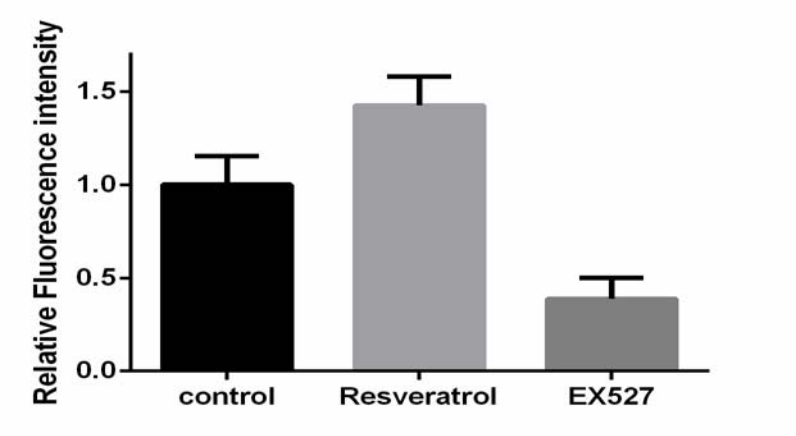


Figure S12 Relative fluorescence intensity of Neonatal cardiomyocytes incubated with TPE-GK(Ac)YDD in the presence or absence of activator resveratrol (20 μM) and inhibitor EX527 (20 μM). (n=6)

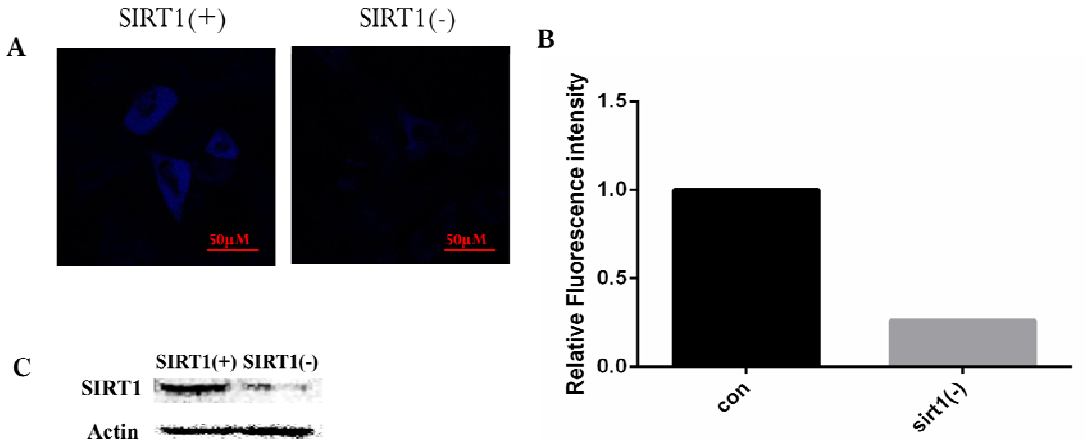


Figure S13 (A) Fluorescence images of different MSC incubated with TPE-GK(Ac)YDD (B) Relative fluorescence intensity of WT rMSC and SIRT1$^{-/-}$ rMSC calculated by Image J. (n=4) (C) The expression of SIRT1 in WT rMSC and SIRT1$^{-/-}$ rMSC by Western Blot.