Supporting information for

A fluorescent peptidyl substrate for visualizing peptidyl-prolyl *cis*/*trans* isomerase activity in live cells

1. Materials and methods

Reagents and apparatus

All chemicals were from commercial supplies and used without further purification. 6-(Dimethylamino)-2-naphthoic acid was prepared as reported.[1] L-phenylalanine *p*-nitrophenyl amide was synthesized according to literature procedures.[2]

^{1}H NMR spectra were obtained on a Bruker 500 Fourier transform spectrometer (500 MHz) at 25 °C. ^{13}C NMR spectra were recorded on a Bruker 500 Fourier transform spectrometer (125 MHz) spectrometer. All NMR spectra were calibrated using the residual solvent (CD_3OD) as internal reference (^{1}H NMR = 3.31, ^{13}C NMR = 49.00). All chemical shifts were reported in parts per million (ppm) and coupling constants (*J*) in Hz. The following abbreviations were used to explain the multiplicities: d = doublet, t = triplet, m = multiplet. High resolution mass spectra (HRMS) were measured on an Agilent 6224 TOF LC/MS spectrometer using ESI-TOF (electrospray ionization-time of flight). Fluorescence spectra were measured on an Agilent Cary Eclipse Fluorescence Spectrophotometer.

Probe synthesis

The key intermediate for **PPI-P**, 6-(dimethylamino)-2-naphthoic acid-Ala-Ala-Pro-OH was synthesized employing *N*-9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acid derivatives by standard carbodiimide (DIC)/1,2,3-Benzotriazol (HOBt) coupling on a chlorotrityl chloride resin. After being cleaved of the fully protected peptide form the resin, it was condensed with *L*-phenylalanine *p*-nitrophenyl amide and then purified by preparative HPLC to yield pure **PPI-P** as a brown powder which was characterized by both NMR spectra and high resolution mass analysis.

^{1}H NMR (500 MHz, CD_3OD) δ 8.35 (d, *J* = 1.4 Hz, 1H), 8.18 – 8.11 (m, 2H), 7.93 (d, *J* = 9.1 Hz, 1H), 7.88 (dd, *J* = 8.6, 1.7 Hz, 1H), 7.80 (d, *J* = 9.2 Hz, 1H), 7.76 – 7.70 (m, 1H), 7.44 (d, *J* = 9.0 Hz, 1H), 7.39 – 7.14 (m, 6H), 4.70 – 4.61 (m, 2H), 4.38 (dd, *J* = 8.4, 5.3 Hz, 1H), 3.83 – 3.67 (m, 1H), 3.61 (m, 1H), 3.30 – 3.26 (m, 1H), 3.26 – 3.22 (m, 1H), 3.22 – 3.17 (s, 6H), 3.10 (dd, *J* = 13.8, 8.4 Hz, 1H), 2.17 – 2.07 (m, 1H), 1.99 – 1.89 (m, 2H), 1.81 (m, 1H), 1.58 – 1.48 (m, 3H), 1.37 (m, 3H).

^{13}C NMR (126 MHz, CD_3OD) δ 175.10, 174.31, 173.73, 172.15, 170.00, 148.05, 145.58, 144.68, 138.19, 137.34, 131.92, 130.70, 130.34, 129.53, 129.09, 128.23, 127.92, 125.98, 125.61, 125.31, 120.60, 120.12, 118.51, 61.95, 57.07, 51.06, 48.87, 48.57, 43.18, 38.24, 30.29, 25.99, 17.99, 16.91. (Signals appear in pairs due to *cis/trans* isomers and presented here was the data of the major one).

ESI-HRMS (*m/z*): $[M+H]^+$ calc'd. for $C_{39}H_{44}N_7O_7$: 722.3302, found 722.3333.

PPI-P fluorescence spectra in the presence of various lysates

PPI-P was dissolved in dry DMSO to make a stock solution of 5 mM. Aloquits of the stock solution were diluted with PBS (10 mM, pH 7.4) to 5 μM and treated with lysates from various cell lines (20 μl) or different amounts of lysates from N2a cells. After an incubation time of 10 min, the spectra were recorded with excitation at 366 nm. The slit width of the fluorescence spectrometer was 2.5 nm for excitation and 5 nm for emission except otherwise indicated.

Recombinant protein

Recombinant protein Pin1 was purchased from Abcam Inc (Catalog, ab51230). Recombinant protein FKBP25 was modified by PCR and subcloned into pET-28a-c(+) vectors (Clontech). The fusion construct was transformed into *Escherichia coli* BL21(DE3) and induced the expression of FKBP25 by addition of isopropyl *β*-D-1-Thiogalactopyranoside (1 mM). The lysate of the FKBP25 products was purified by Amicon Pro Purification System (ACS501012, Massachusetts, USA). The activity of the recombinant FKBP25 was detected by chymotrypsin-coupled assay.

Culture of cells

EA.hy926, human embryonic kidney (HEK)-293, mouse neuroblastoma 2a (N2A) cells and human brain vascular pericyte (HBVP) cells were used in the study. Briefly, the cells were grown in DMEM with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere containing 5% CO_2. In particular, HBVPs were cultured in pericyte growth medium (containing growth factors, hormones, and proteins, ScienCell Research Laboratories; Catalog #1252). Experiments were performed with cells as described previously.[3]

Cell fractionation.

Wash cells three times before collecting. Then add appropriate lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 100 μg/ml PMSF, pH 7.5) into the eppendorf tubes. Keep the tubes on ice for 30 min and vortex it every 10 min. The tubes were centrifuged for 10 min at 12,000 g and preserved the supernatant at -80 °C. Furthermore, protein concentration of the lysate was determined by spectrophotometry and was diluted to 1 μg/μl of protein concentration with lysis buffer before use.

Fluorescence assay for PPIases with a microplate reader

Added 50 μL HEPES buffer (100 mM, pH 8.0), 1 μL probe **PPI-P** (5 mM) and indicated volume of lysis buffer or recombinant protein into the well plate. Diluted the total system to 100 μL with ddH_2O. Then put the 96-well plate (black) in the microplate reader of multi-wavelength measurement system (SynergyMx M5, Molecular Devices). Shake the well plate for 5 s before recording the emission at λ_{em} 460 nm with λ_{ex} 366 nm, and collected data every 20 s for 500 s.

Live cell imaging

EA.hy926 cells were grown as described for fluorescence trafficking.[4,5] The cells were infected with lentiviral vectors encoding EGFP or FKBP25-EGFP (Obio Technology) before imaging. Cells were cultured in glass bottom dishes till reaching desired density. The dish was then mounted on Olympus IX-81 confocal microscope (YOKOGAWA, Japan) equipped with a temperature controller to keep the temperature at 37°C, a ×60 oil-immersion lens, a polychrome IV light source (Till Photonics), a 505 DCXR beam splitter, and a CCD camera (ANDOR iXon3). DMEM (2 mL) with 10% FBS containing probe **PPI-P** (2.5 μM) was warmed in a culture incubator for 5 min at 37°C, and then was used to displace the medium in the dish. Images were captured as time series using diode UV laser for probe (λ_{ex} 405 nm, λ_{em} 420-480 nm) and Argon laser for EGFP (λ_{ex} 488 nm, λ_{em} 500-550 nm).

Cell viability analysis

The number of viable cells was determined using Cell Counting kit 8 (CCK-8; Dojindo Laboratories, Japan). First, EA.hy926 cells were cultured in 96-well plates with a density of 5000 cells/well. On the second day, the culture medium was replaced with 100 µl DMEM containing different concentration of **PPI-P** (0.1, 1, 2.5, 10, 50 µM). Then, CCK-8 reagent (10 µl) was added into each well and the plate was kept at 37°C for 2 h. Finally, the absorbance at 450 nm was detected using the Beckman Coulter DTX 880 Multimode Detector.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's *post hoc* test was used for cell viability assay. Two-way ANOVA followed by *post hoc* Sidak's multiple comparison test was used for probe intensity of fluorescence analysis. The live cell images were evaluated using Image J software (NIH, Bethesda, MD). Statistical analyses were carried out by using GraphPad Prism 6 (GraphPad Software), and $p < 0.05$ was indicated statistically significant.

References:

1. A. S. Rao, D. Kim, T. Wang, K. H. Kim, S. Hwang and K. H. Ahn, *Org Lett*, 2012, **14**, 2598-2601.
2. J. Dong, Y. Wang, Q. Xiang, X. Lv, W. Weng and Q. Zeng, *Adv Synth Catal*, 2013, **355**, 692-696.
3. Q. Jiang, Y. Gao, C. Wang, R. Tao, Y. Wu, K. Zhan, M. Liao, N. Lu, Y. Lu, C. S. Wilcox, J. Luo, L. H. Jiang, W. Yang and F. Han, *Antioxid Redox Signal*, 2017.
4. X. Li, R. R. Tao, L. J. Hong, J. Cheng, Q. Jiang, Y. M. Lu, M. H. Liao, W. F. Ye, N. N. Lu, F. Han, Y. Z. Hu and Y. H. Hu, *J Am Chem Soc*, 2015, **137**, 12296-12303.
5. L. J. Hong, Q. Jiang, S. Long, H. Wang, L. D. Zhang, Y. Tian, C. K. Wang, J. J. Cao, R. R. Tao, J. Y. Huang, M. H. Liao, Y. M. Lu, K. Fukunaga, N. M. Zhou and F. Han, *Mol Neurobiol*, 2016, **53**, 1237-1246.

2. Supplementary figures

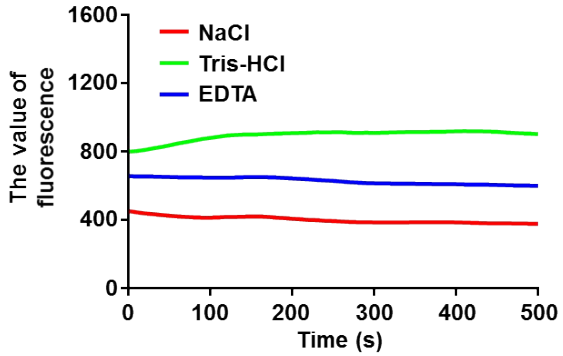


Fig. S1 The lysis buffer solutions have no effect on probe **PPI-P** fluorescence. The fluorescence of **PPI-P** showed no change towards indicated solutions at given time. The recording was initiated by the addition of 1 µL **PPI-P** (5 mM) to a solution containing 50 µL HEPES buffer (100 mM, pH 8.0), 39 µL ddH_2O and 10 µL indicated lysis buffer solutions.

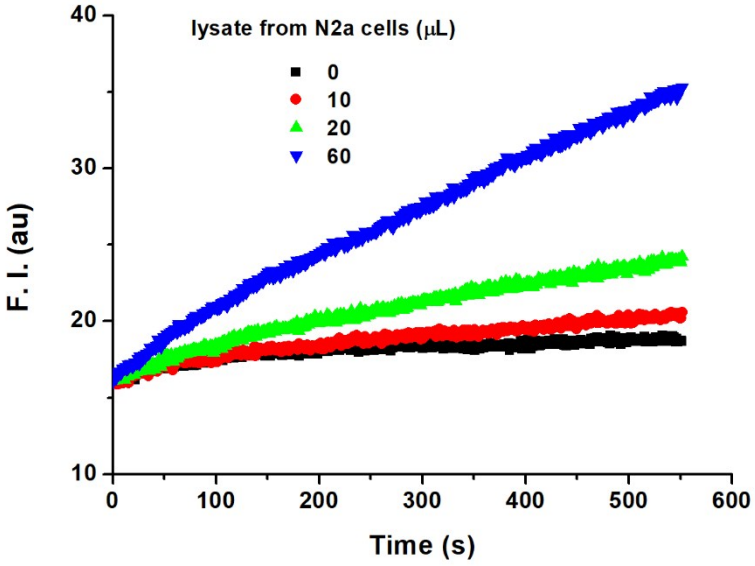


Fig. S2 Dose-dependent responses of **PPI-P** fluorescence on lysates from N2a cells. Aliquots (1 mL) of a solution of **PPI-P** (5 μM) in PBS (10 mM, pH 7.4) were treated with various amounts of N2a lysate (total protein concentration of this lysate: 1 μg/μL). The emission at 460 nm was monitored against incubation time with excitation at 363 nm. Data were collected on an Agilent Cary Eclipse Fluorescence Spectrophotometer at ambient temperature.

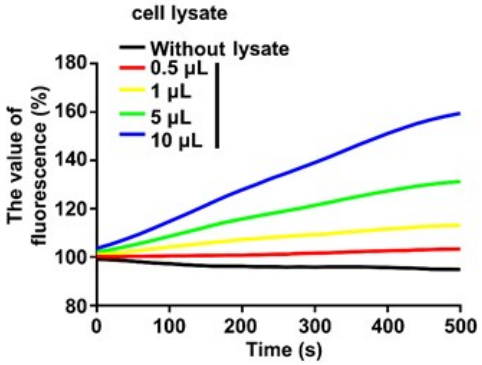


Fig. S3 Dose-dependent responses of **PPI-P** fluorescence on lysates from HEK-293 cells. **PPI-P** fluorescence increased in a lysate dose-dependent way. The assay was detected with 50 μL HEPES buffer (100 mM, pH 8.0), 1 μL probe **PPI-P** (5 mM) and indicated volume of lysate from HEK-293 cells (total protein concentration of this lysate: 1 μg/μL). The total system was increased to 100 μL by the addition of ddH_2O. Data was obtained on a microplate reader of multi-wavelength measurement system (SynergyMx M5, Molecular Devices).

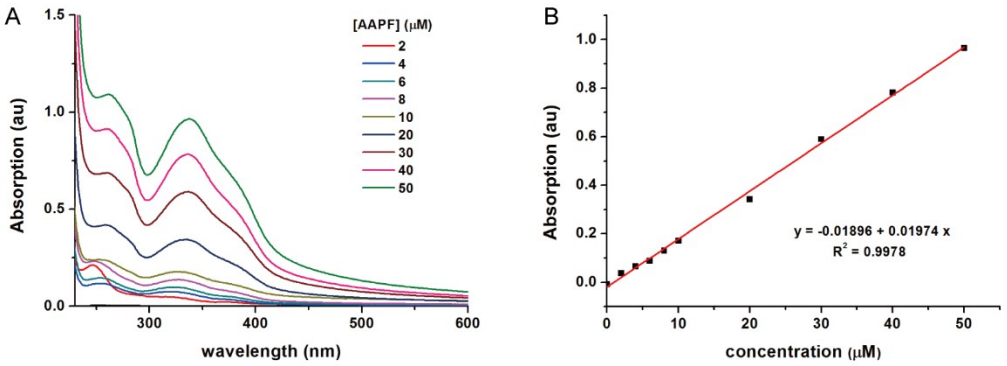


Fig. S4 Solubility test of **PPI-P** in PBS (pH 7.4, 10 mM). A) UV-Vis absorption spectra of **PPI-P** in PBS. B) Plot of the absorption intensity at 337 nm versus the concentration of **PPI-P** (0-50 μM) gave a linear correlation.

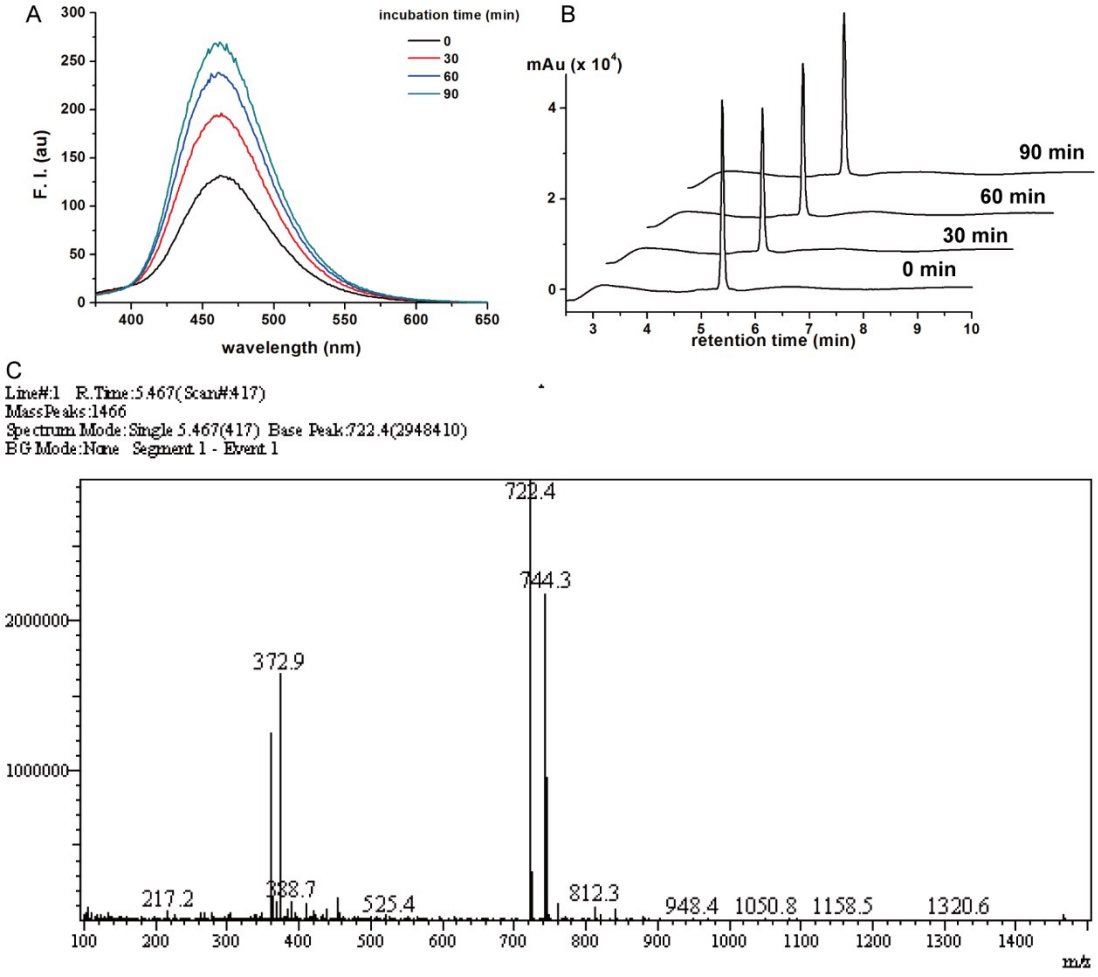


Fig. S5 Stability of **PPI-P** in lysates from HEK 293 cells. A) **PPI-P** (10 μM) in PBS (pH 7.4, 10 mM) was incubated with cell lysis (40 μL, 3μg/μL), and the fluorescence spectra of the mixture was recorded at indicated time on an Agilent Cary Eclipse Fluorescence Spectrophotometer with excitation at 363 nm, and slit width 2.5 nm for excitation and 10 nm for emission. B) the mixture at indicate time was analyzed by LC-MS and the LC traces showed no new peak other than PPI-P. C) Mass spectra of the peak in Fig B showed its identity to be **PPI-P**.

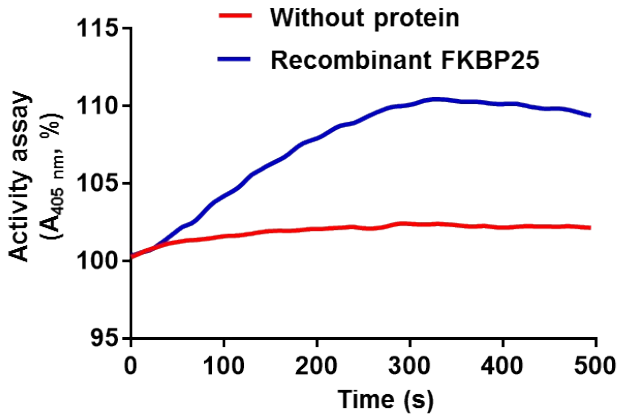


Fig. S6 Activity of recombinant FKBP25 by chymotrypsin-coupled assay. Traces shown were the time-dependent changes of absorbance of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide peptide in response to recombinant FKBP25 treatment. The assay was performed in 35 mM Hepes buffer (pH 8.0) with 100 μM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide peptide and 250 μg/mL α-chymotrypsin, with or without recombinant protein FKBP25 treatment. The release of *p*-nitroanilide was monitored every 5 s for 500 s at 405 nm with the Beckman Coulter DTX 880 Multimode Detector.

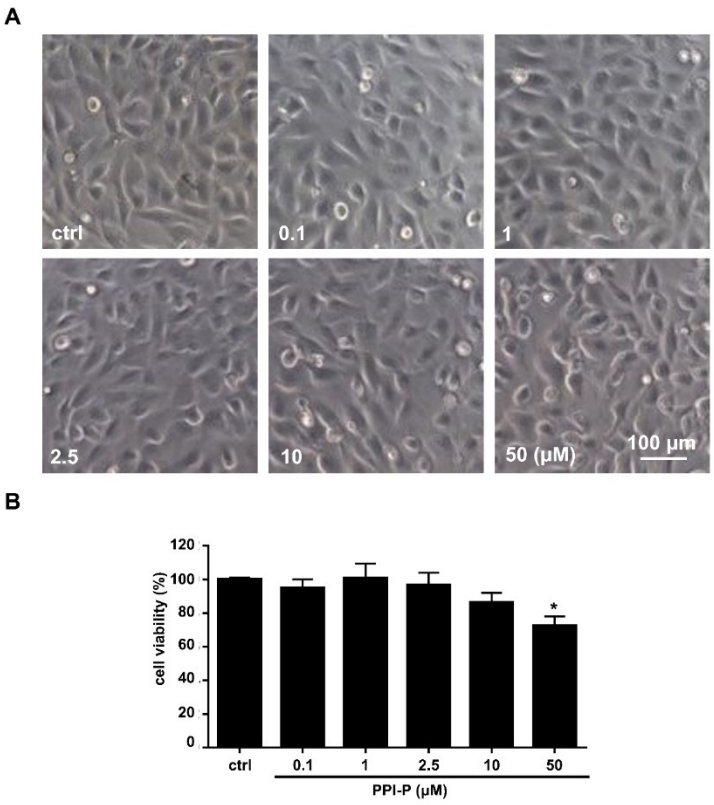


Fig. S7 Effects of **PPI-P** on cell viability as examined by CCK-8 assay. (A) The morphology of EA.hy926 cells was recorded after incubation with **PPI-P** (0.1, 1, 2.5, 10, 50 μM). (B) The viability of EA.hy926 cells in the presence of **PPI-P** was detected by CCK-8 assay. No significant change of cell viability was observed until **PPI-P** was used at a high concentration of 50 μM. Assays were performed in triplet. **p* < 0.05 versus control. Ctrl, control.

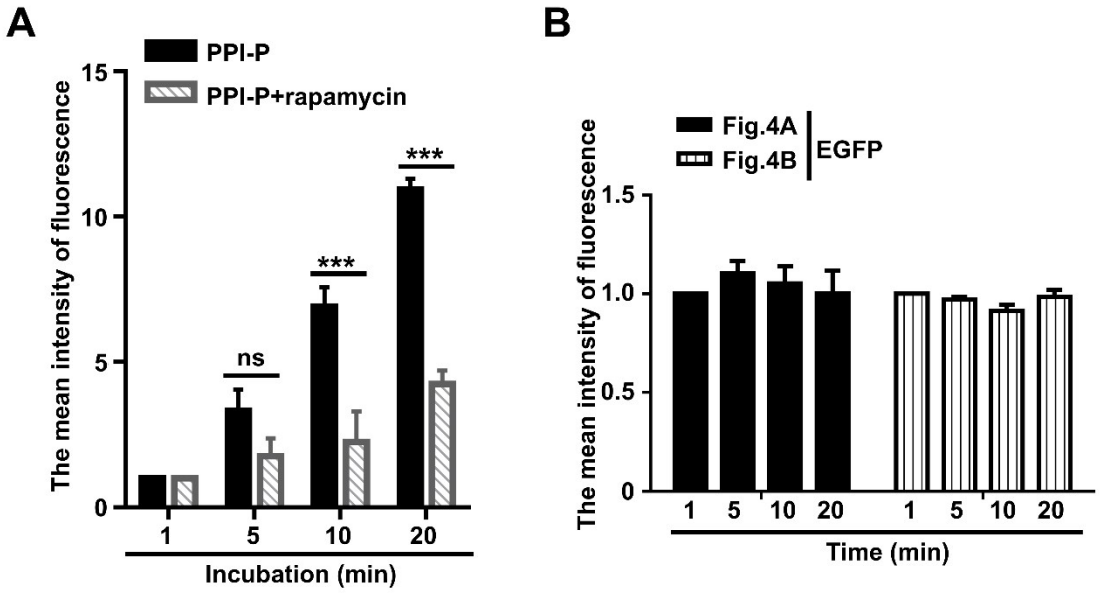


Fig. S8 Quantitative analysis of probe **PPI-P** (A, λex 405 nm) and EGFP (B) fluorescence mean intensity in live cells treated with or without rapamycin at different time point as shown in Fig. 4. There is no statistically difference with one-way ANOVA followed by Tukey's post hoc test in B. Data shown were the average from three independent experiments. $^{***}p < 0.001$; ns, no significant.

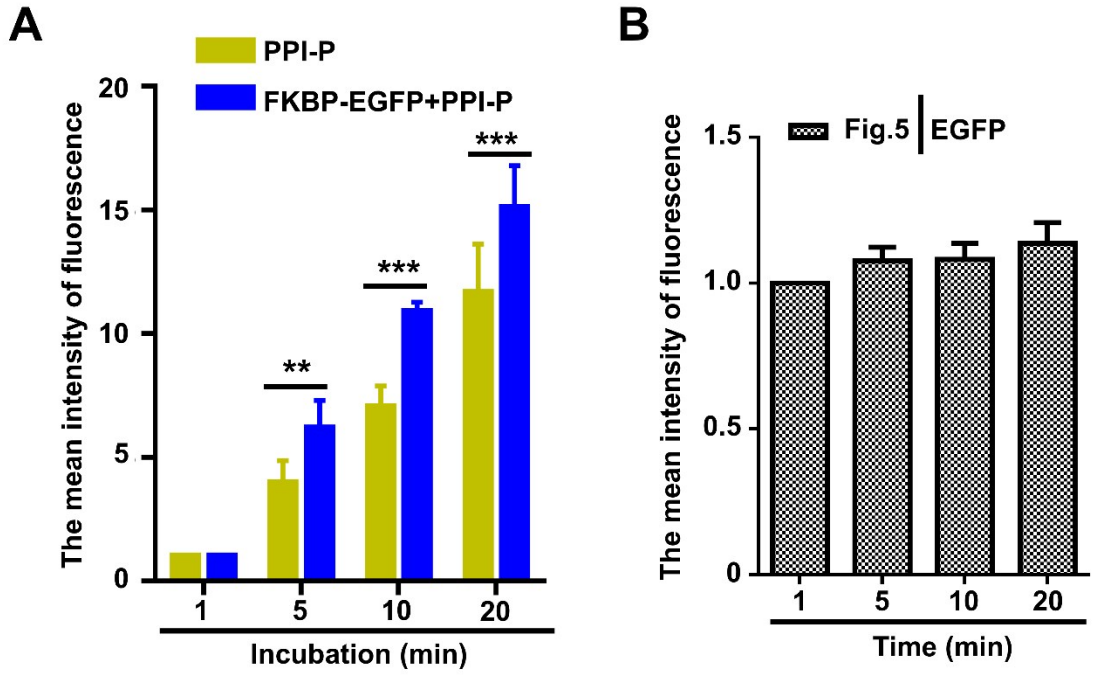
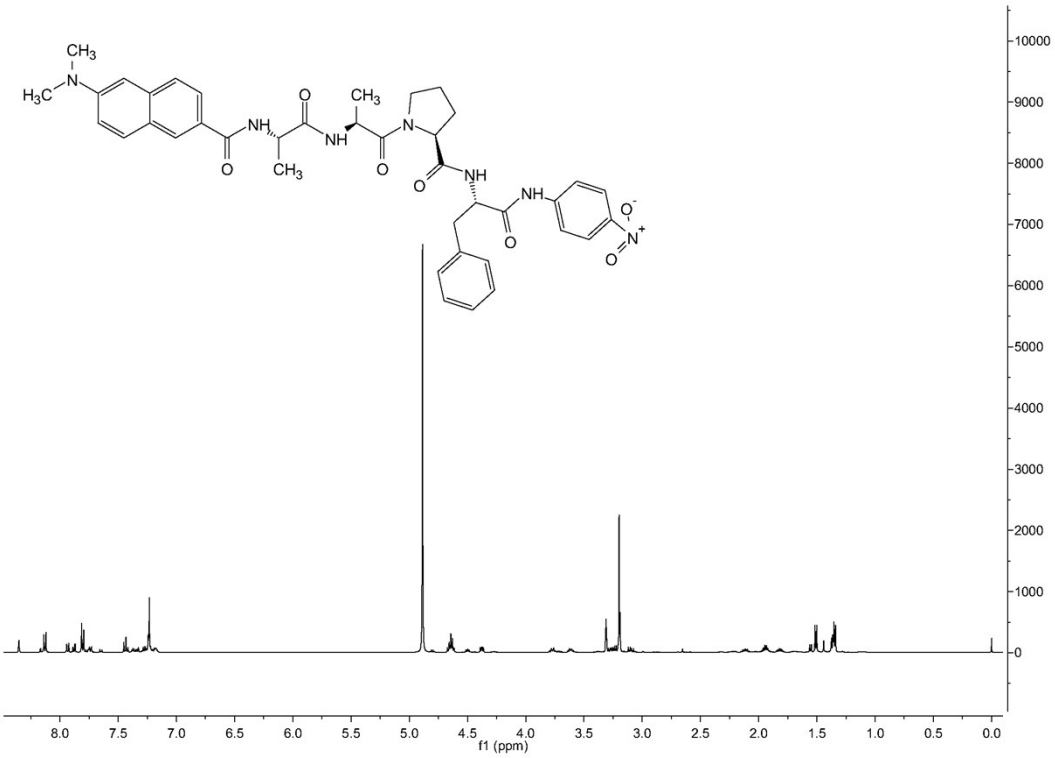
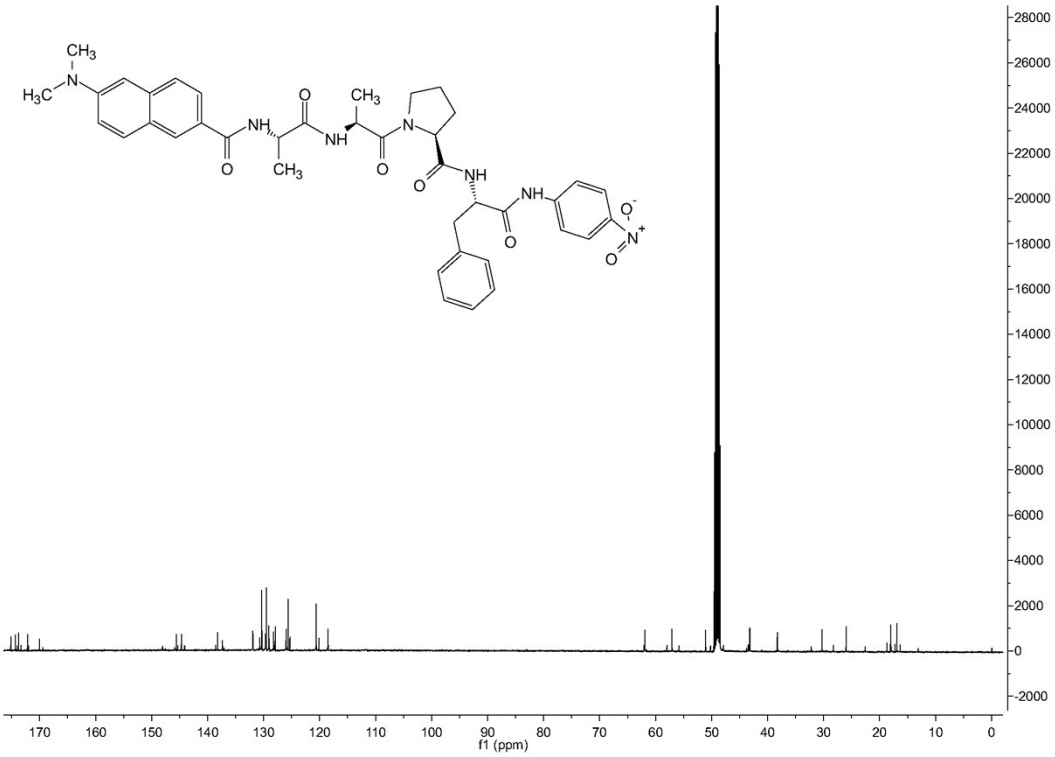


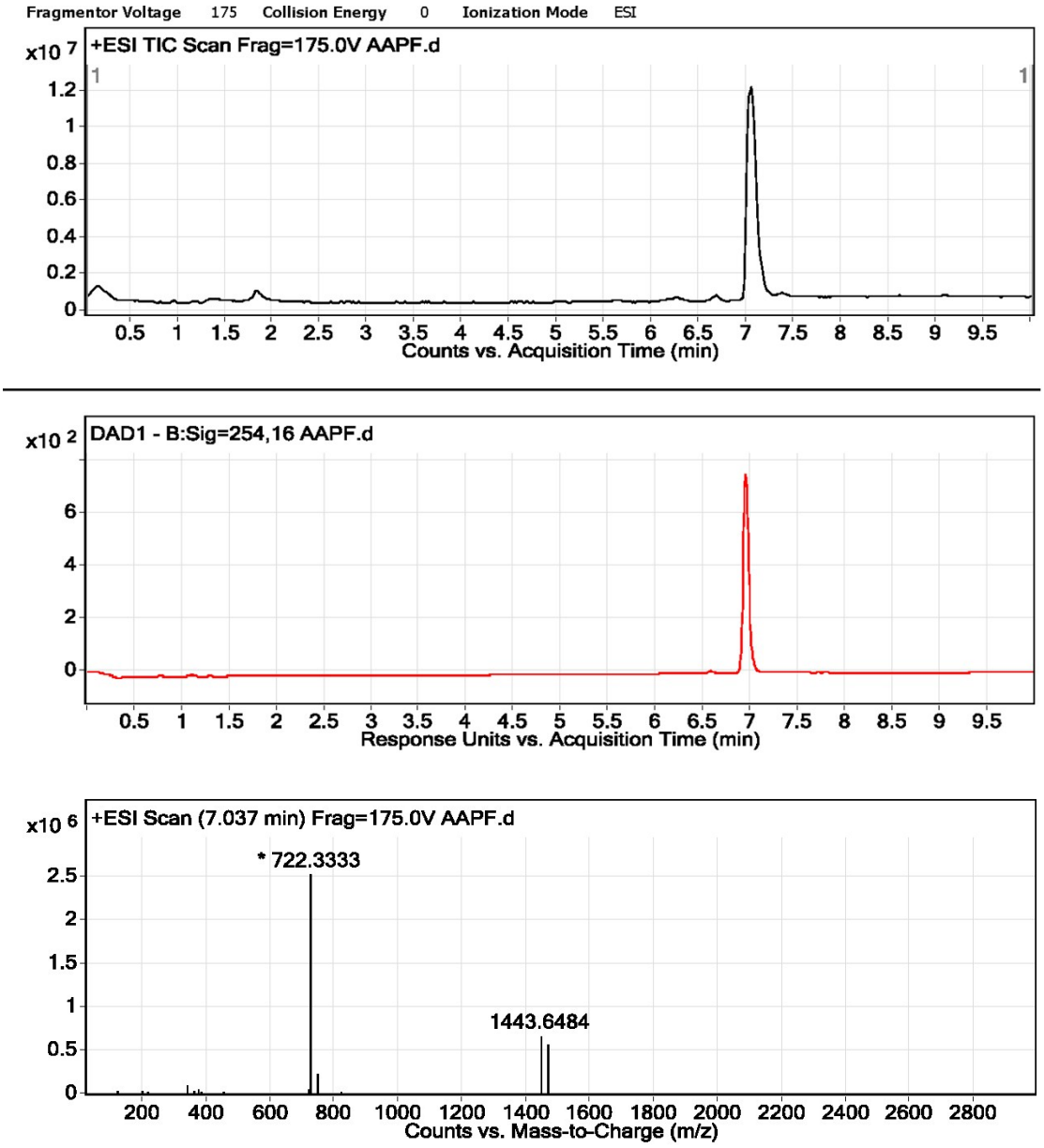
Fig. S9 Quantitative analysis of probe **PPI-P** (A, λex 405 nm) and EGFP (B) fluorescence intensity in live cells without (yellow) or with (white) FKBP25 overexpression at different time point as shown in Fig. 5. There is no statistically difference with one-way ANOVA followed by Tukey's post hoc test in B. The results were presented in the bar graph from three independent experiments. $^{**}p < 0.01$. $^{***}p < 0.001$.



^{1}H NMR trace of PPI-P



^{13}C NMR trace of PPI-P



LC-HRMS trace of PPI-P