Supporting Information

A ratiometric fluorescent probe for endogenous biological signaling molecule superoxide anion detection and bioimaging during tumor treatment

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Content

1. Experimental section

Materials and methods: TP-Tfs (1.0 mM, 10 mL) was prepared in DMSO and stored at 4 °C in darkness. Stock solutions of glutathione (GSH), vitamin c (Vc), vitamin E (Ve), were prepared to desired concentrations when needed. NO_2^- was generated from $NaNO_2$. $O_2^{-\cdot}$ was created by the KO_2. OCl^- was standardized at pH 12 ($\varepsilon_{292\ nm}$ = 350 $M^{-1}cm^{-1}$).[1] H_2O_2 was determined at 240 nm ($\varepsilon_{240\ nm}$ = 43.6 $M^{-1}cm^{-1}$). ·OH was generated by Fenton reaction between Fe^{II}(EDTA) and H_2O_2 quantitively, and Fe^{II}(EDTA) concentrations represented ·OH concentrations. *Tert*-butylhydroperoxide (*t*-BuOOH) and cumene hydroperoxide (CuOOH) could also be used to induce ROS in biological systems. The $ONOO^-$ source was the donor 3-Morpholinosydnonimine hydrochloride (SIN-1, 200 μmol/mL).[2] NO was generated in form of 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 100 μM/mL).[3] Methyl linoleate (MeLH) and 2,2'-azobis-(2,4-dimethyl)valeronitrile (AMVN) were used to produce MeLOOH.[4,5] $^1O_2$ was generated from 3,3'-(naphthalene-1,4-diyl) dipropionic acid.[6] ROO• was generated from 2,2'-azobis(2-amidinopropane) dihydrochloride.[7] Angeli's salt (a HNO donor) was prepared as reported by King and Nagasawa and stored dry at - 20°C in a refrigerator.[8] LPS, PMA, rotenone, malonic acid, FCCP, antimycin A, 2-ME, tiron, TEMPO, DDP, Vc, Ve and cum were prepared when were needed. All the reagents were obtained from Aladdin (USA). All other chemicals were from commercial sources and of analytical reagent grade, unless indicated otherwise. Thin-layer chromatography (TCL) was performed on silica gel plates. Silica gel P60 (SiliCycle) was used for column chromatography (Hailang, Yantai) 200-300 mesh. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. The following rabbit antibodies were purchased from Cell Signaling Technology (β-actin: #4970, 1:1000; cytochrome *c*: #1940, 1:1000; Bax: #5023, 1:1000; Bcl-2: #4223, 1:1000). The following cell dyes were purchased from Thermo Fisher Scientific (JC-1: #T3168; Fluo 4-AM: #F14201). Annexin V/7-AAD Apoptosis Detection Kit was purchased from BD Biosciences (#559763). ROS probe 2,7-dichlorodihydrofluorescein diacetate was purchased from Thermo Fisher Scientific. Ultrapure water was used throughout.

Apparatus: Absorption spectra were determined by UV-vis spectrometer evolution 200 (Thermo Scientific). Fluorescence spectra were collected on a HORIBA Scientific Fluoromax-4 spectrofluorometer. All pH measurements were performed by a basic pH-Meter PH-3C digital pH-meter (Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. ^1H NMR spectra were taken on a Bruker spectrometer. The one-photon fluorescence images of cells were acquired using a LTE confocal laser scanning microscope (Olympus FV1000 confocal laser-scanning microscope). The two-photon fluorescence images of cells were collected using a two-photon laser confocal microscope (Zeiss LSM 880). Flow cytometry data were collected by BD Biosciences FACSAria. Ultrathin sections were cut using Leica EM UC7. Absorbance was measured in a TECAN infinite M200 PRO microplate reader in the MTT assay.

Spectrophotometric Measurements: The fluorescence and absorption spectra were measured in 10 mM HEPES (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid) solution. The pH gradient of HEPES buffer solution from 4.0 to 10.0 was achieved by adding different volumes of HCl or NaOH solution. Absorption spectra were collected with 1.0-cm glass cells. Spectral detection was performed as following: 1 mL various concentrations (0 μM - 20 μM) of $O_2^{-\cdot}$ was added into a 10.0 mL color comparison tube and diluted to 10.0 mL with HEPES buffer. Then probe (10 μM) was added. All spectroscopic experiments were carried out at room temperature.

Cell culture: Human neuroblastoma (SH-SY5Y) cells and human lung carcinoma (A549) cells

were obtained from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). SH-SY5Y cells were cultured with RPMI-1640 supplemented with 10% FBS. And A549 cells were cultured with DMEM supplemented with 10% FBS. All cells were maintained at 37 °C under a humidified atmosphere containing 5% CO_2 and 95 % air. The cells were passaged by scraping and seeding on 20 mm Petri-dishes according to the instructions from the manufacturer.

Flow Cytometry Analysis: The cells were cultured at 2.0×10^5 cells/well in 6-well plates, and then the cells were treated as described in the paper. After harvest, cells were washed for three times with PBS, and then analyzed by flow cytometry.

MTT assay: A549 cells and SH-SY5Y cells were grown in an atmosphere of 5% CO_2 and 95% air at 37 ^oC. The cells were seeded into 96-well plates at a density of 1×10^5 cells/mL in 100 μL medium and allowed to adhere for 24 hours. Then the cells were incubated for 24 h at 37 °C in a 5% CO_2/95% air upon different concentrations probe of 0 μM to 70 μM respectively. Plates were then washed with PBS before MTT solution was added to each well in an atmosphere of 5% CO_2 and 95% air. The plate was shaken and the absorbance was measured at 490 nm using a microplate reader (TECAN infinite M200pro).

Western blot analysis: Kidney was homogenized and protein was extracted with 200 μl RIPA lysis buffer containing 2 μl PMSF (Solarbio, China). Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Bioworld Technology, USA). Protein samples were prepared after centrifugation at 12,500 g at 4 ^oC for 15 min. Then, the protein was mixed with loading buffer (4:1) and heated at 95 ^oC for 15 min. For Western blotting analysis an equal amount of protein (70 μg) was loaded in each well and subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred from the gel to polyvinylidinene fluoride (Millipore, Bedford, MA, USA) membranes and blocked in 7% non-fat dry milk prepared in 1×TBST. The membranes were incubated with the primary antibodies overnight at 4 ^oC. The following primary antibodies were used: β-actin, PARP, cytochrome *c*, Bax and Bcl-2. After washing primary antibodies with 1×TBST, the membranes were incubated with appropriate secondary antibodies for 1 h at room temperature. The membranes were developed using Super Enhancer ECL Kit (Shanghai Novland, China). The results were analyzed by Image J to acquire the grey value of every bond.

H&E Staining: Kidneys from model mice were excised. The tissues were embedded in paraffin after fixed with 10% formaldehyde. The treated kidneys were cut and dewaxed. Then the sections were dehydrated with graded ethanol series and then they were washed with distilled water. The kidney sections were stained with hematoxylin and eosin (H&E) for observation.

Ethics statement: All surgical procedures were conducted in conformity with the Care and Use of National Guidelines for the laboratory animals, and experimental protocols were approved by the Institutional Animal Care and Use Committee in Binzhou Medical University, Yantai, China. Approval Number: No.BZ2014-102R.

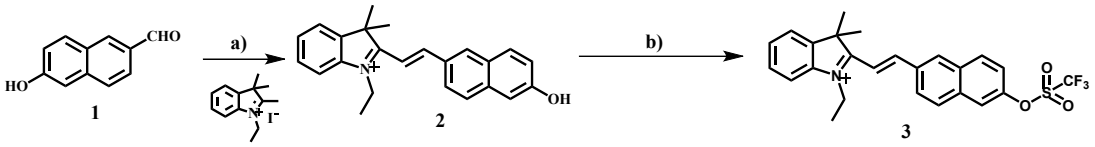
Establishment of DDP chemotherapy mice model with adjuvant agent:
DDP Chemotherapy group: When the tumor size reached 200 mm^3, DDP (0.5 mg/mL × 0.5 mL) was given by intraperitoneal injection (i.p.) every other day.

Cum supplementary DDP chemotherapy group: When the tumor size reached 200 mm^3, DDP (0.5 mg/mL × 0.5 mL) was given by intraperitoneal injection (i.p.) every other day and cum (50 mg/kg) was given via tail vein injection three times a week.[9]
Vc supplementary DDP chemotherapy group: When the tumor size reached 200 mm^3, DDP (0.5 mg/mL × 0.5 mL) was given by intraperitoneal injection (i.p.) every other day and Vc (1mg/g) was given twice a week.[10]
Ve supplementary DDP chemotherapy group: When the tumor size reached 200 mm^3, DDP (0.5 mg/mL × 0.5 mL) was given by intraperitoneal injection (i.p.) every other day and Ve (1mg/g) was given twice a week.

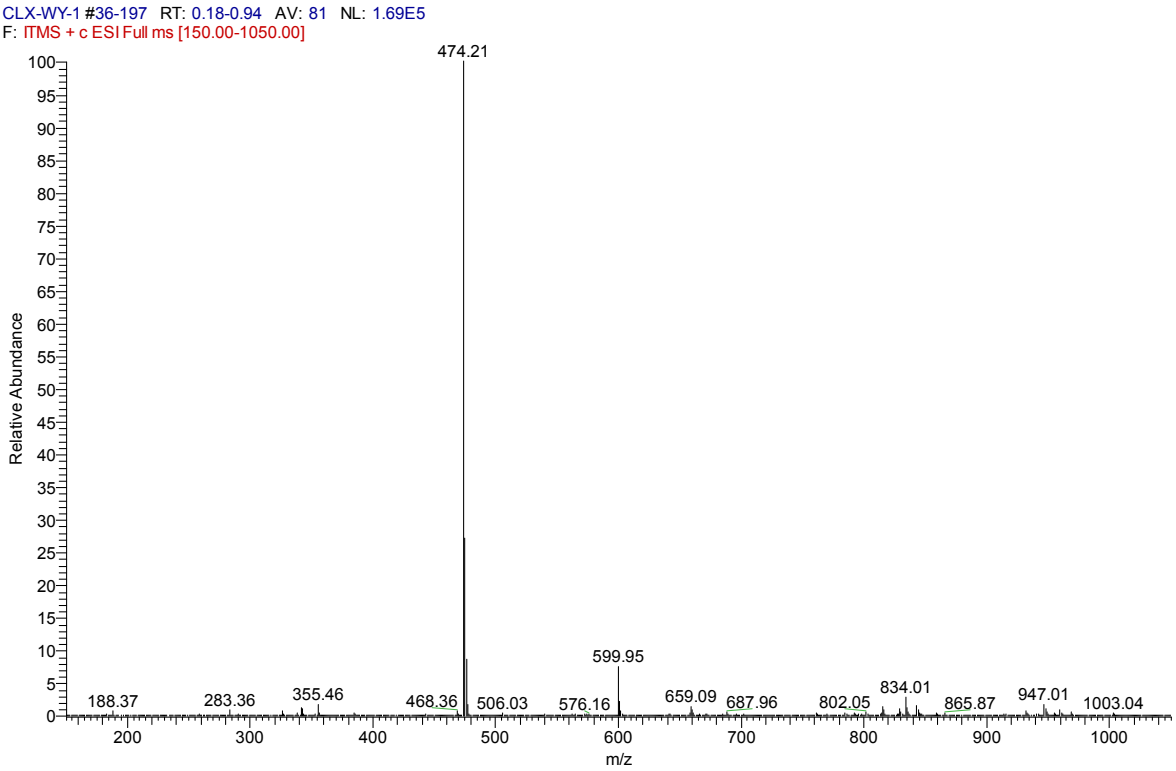
Statistical Analysis: Statistical Product and Service Solutions (SPSS) software was used for the statistical analysis. The error bars shown in the figures represented the mean ± s.d. Differences were determined with a one-way analysis of variance (ANOVA) followed by LSD test. Statistical significance was assigned at $^{*}P<0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Sample size was chosen empirically based on our previous experiences and pre-test results. No statistical method was used to predetermine sample size and no data were excluded. The numbers of animals or samples in every group were described in the corresponding figure legends. The distributions of the data were normal. All experiments were done with at least three biological replicates. Experimental groups were balanced in terms of animal age, sex and weight. Animals were all caged together and treated in the same way. Appropriate tests were chosen according to the data distribution. Variance was comparable between groups in experiments described throughout the manuscript.

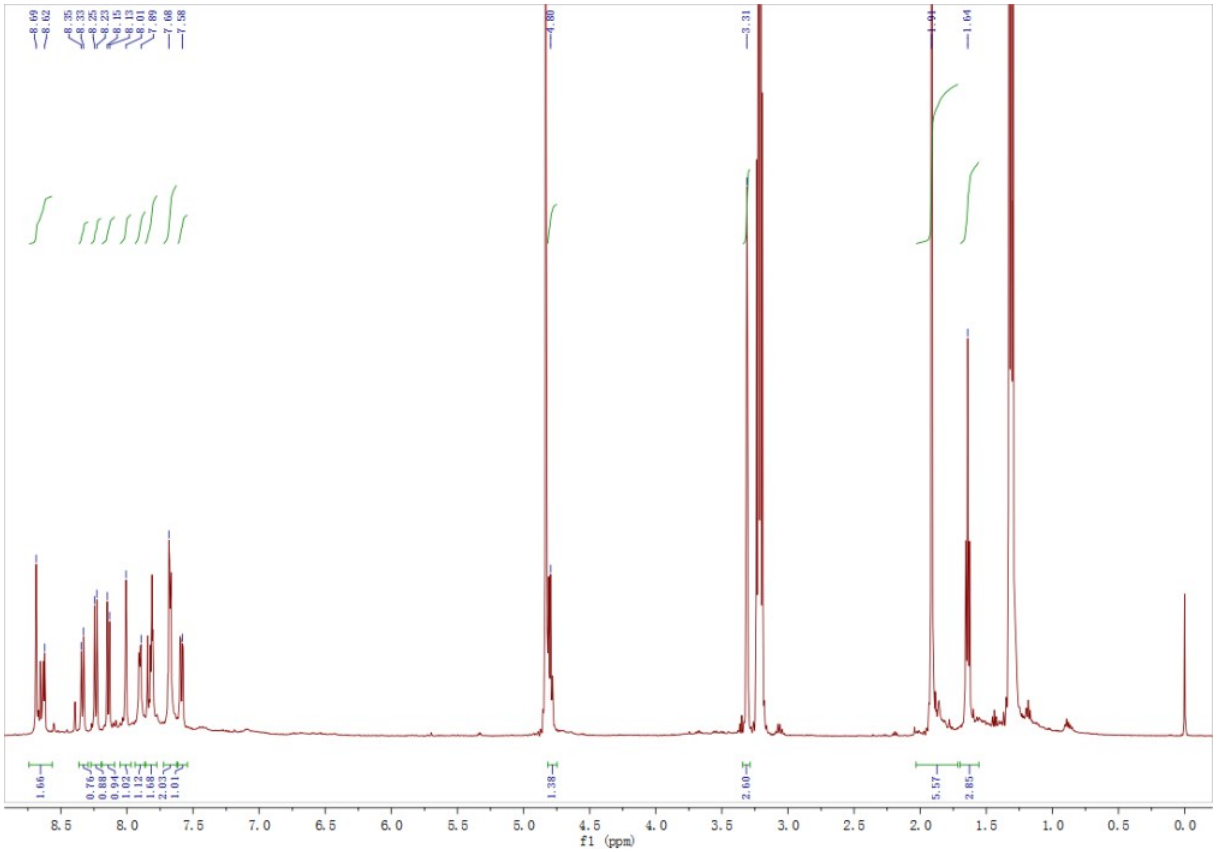
2. Synthesis and characterization of compounds

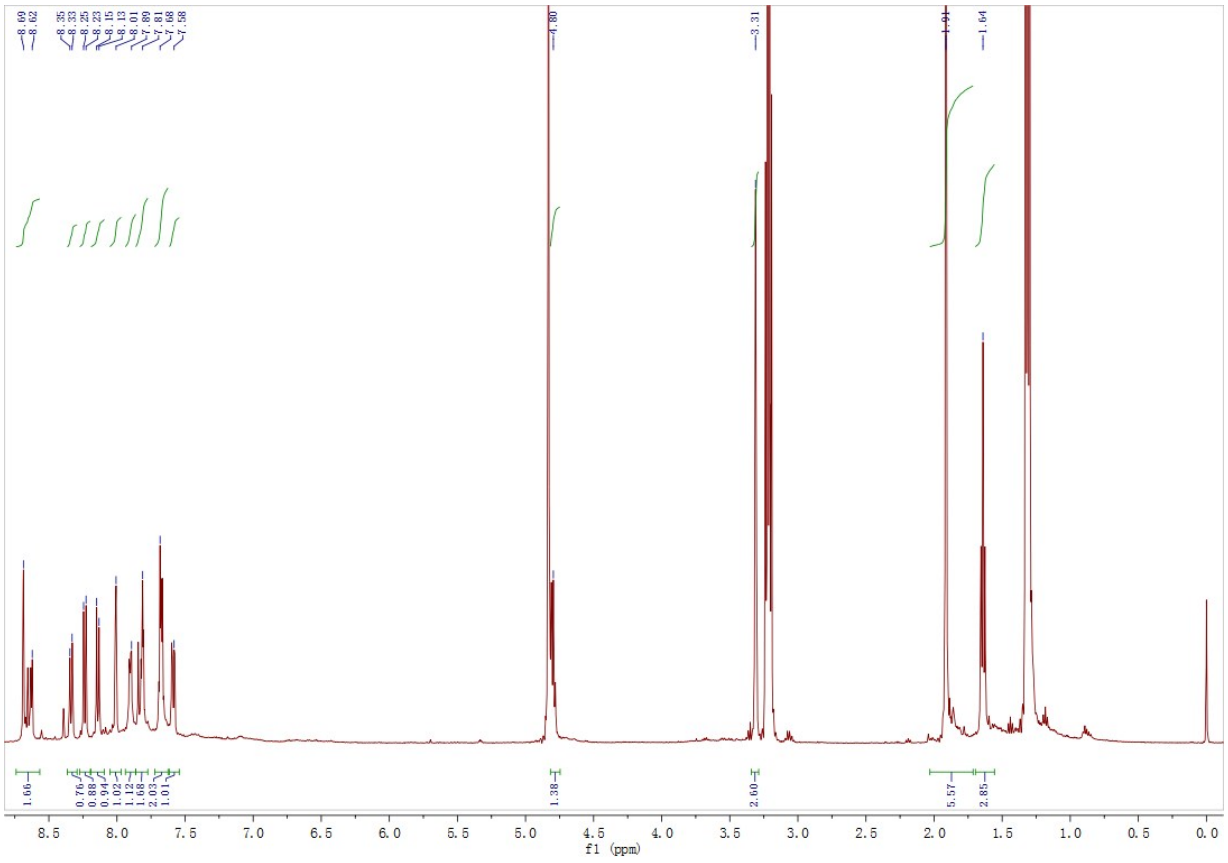
Scheme S1. Synthetic routes for probe TP-Tfs.



Synthesis of compound 2. Compound hemicyanine (1 g, 3.17 mmol) and 6-Hydroxy-2-naphthaldehyde (compound 1, 0.5 g, 2.9 mmol) were dissolved in n-butyl alcohol and benzene (7:3 v/v), refluxed for 4 h. After concentrated, the obtained crude product was purified by silica column chromatography (200 - 300 mesh) to afford an orange product (0.73 g, 73.5%).


CLX-WY-1 #36-197 RT: 0.18-0.94 AV: 81 NL: 1.69E5
F: ITMS + c ESI Full ms [150.00-1050.00]
474.21
599.95
834.01
188.37
283.36
355.46
468.36
506.03
576.16
659.09
687.96
802.05
865.87
947.01
1003.04
Relative Abundance
m/z


f1 (ppm)



3. Absorbance spectroscopic measurements

Absorption spectra were recorded on UV-vis spectrometer evolution 200 (Thermo Scientific).

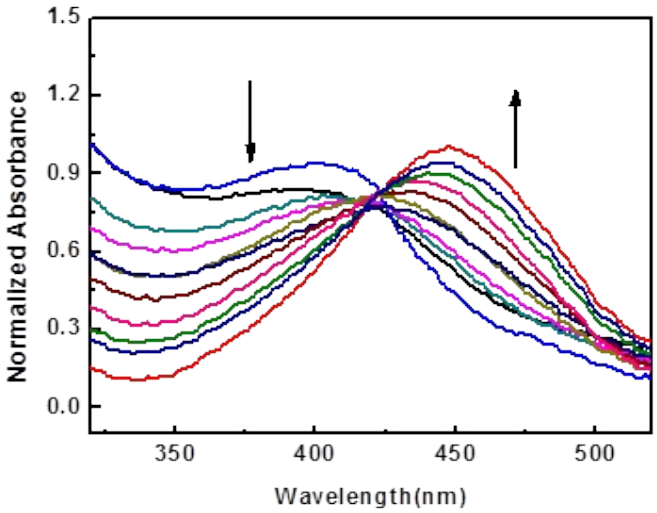


Fig. S1. The absorption spectral of TP-Tfs (10 μM) towards $O_2^{\cdot-}$ (20 μM).

4. Effect of pH values

To apply the probe in complicated system for $O_2^{\cdot-}$ detection, the effect of pH in the range of 4.0-10.0 on TP-Tfs and TP-OH were investigated. It was found that TP-Tfs (10 μM) was hardly affected in the region of 4.0 - 8.0. The reaction product of TP-Tfs, TP-OH (10 μM), was stable during pH range from 4.0 to 8.0. Thus, our probe can function properly at physiological pH.

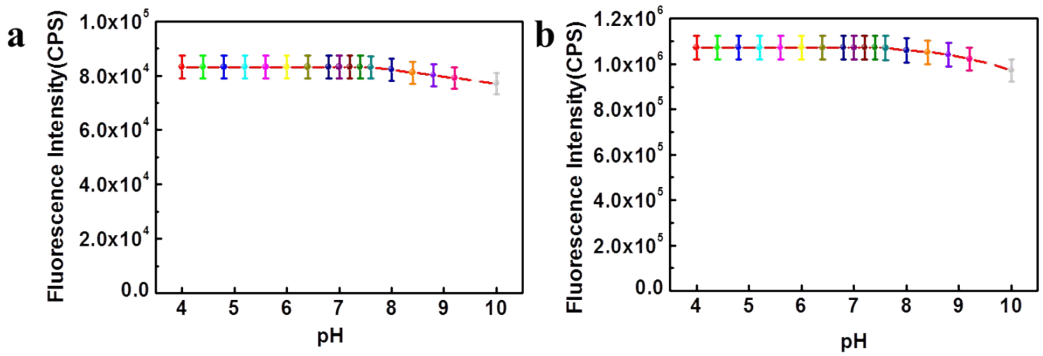


Fig. S2. Fluorescence intensity changes of TP-Tfs (10 μM) (a) and TP-OH (10 μM) (b) at different pH values. The reactions were carried out for 5 min at room temperature in 10 mM HEPES solution.

5. Cytotoxicity assay

To evaluate the cytotoxicity of TP-Tfs and TP-OH, we performed MTT assays on A549 cells and SH-SY5Y cells with concentrations from 0 - 70 μM. The cells were seeded into 96-well plates at a density of 1×10^5 cells/mL in 100 μL medium and allowed to adhere for 24 hours. Subsequently, the cells were cultured with 0 - 70 μM (final concentration) of TP-Tfs and TP-OH at 37 °C, and the cultures were maintained in an atmosphere of 5% CO_2 and 95% air for 24 h. Then MTT solution was added to each well. The plate was shaken and the absorbance was measured at 450 nm using a microplate reader (TECAN infinite M200pro).

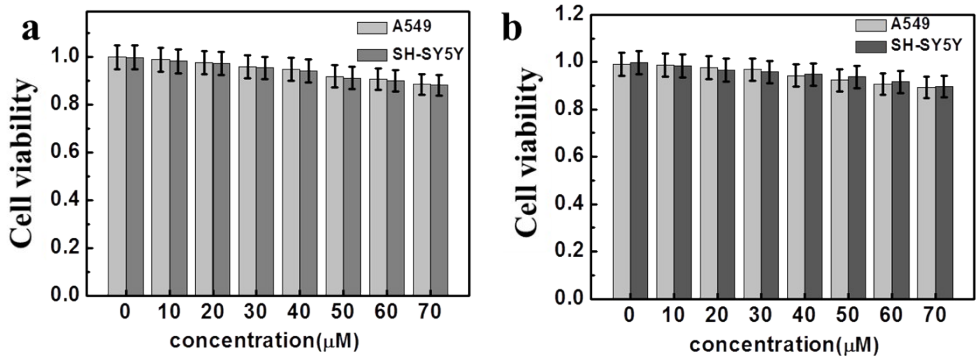


Fig. S3. The cytotoxicity of TP-Tfs (a) and TP-OH (b) at different concentration (0-70 μM) in A549 cells and SH-SY5Y cells. The experiment was repeated five times and the data are shown as mean (±S.D.).

6. Histograms of average ratio fluorescence intensities for Fig 2

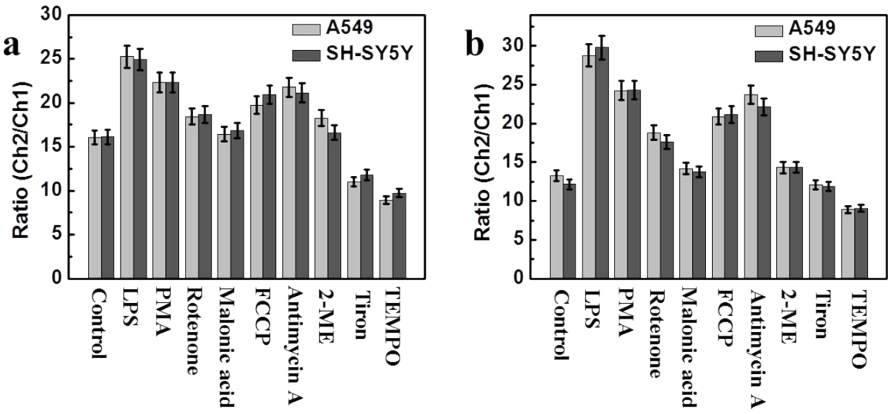


Fig. S4. (a) Histograms of average ratio fluorescence intensities in Fig.2a. (b) Histograms of average ratio fluorescence intensities in Fig.2b.

7. Average ratio intensities analysis for Fig.3

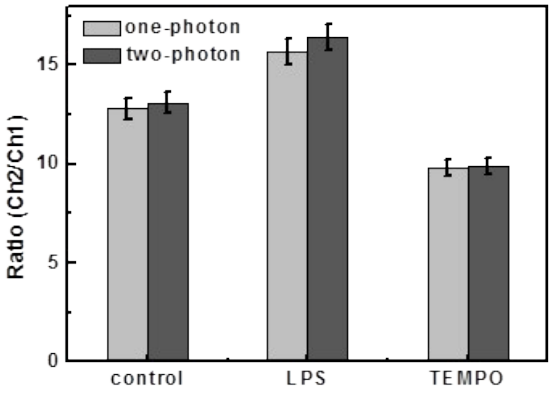


Fig. S5. Average ratio intensities analysis for Fig.3.

8. Intracellular Ca^{2+} detection in Fig.4

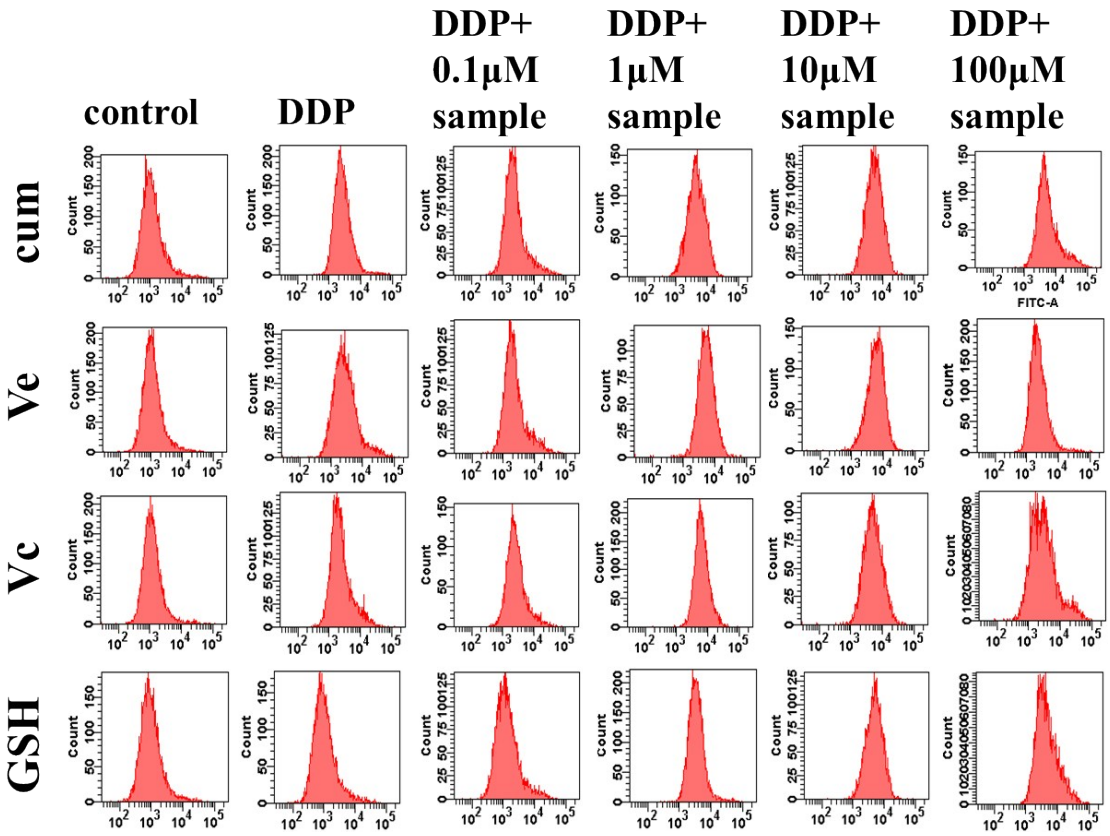


Fig. S6. Intracellular Ca^{2+} detection in Fig.4.

9. Intracellular ROS detection in Fig.4

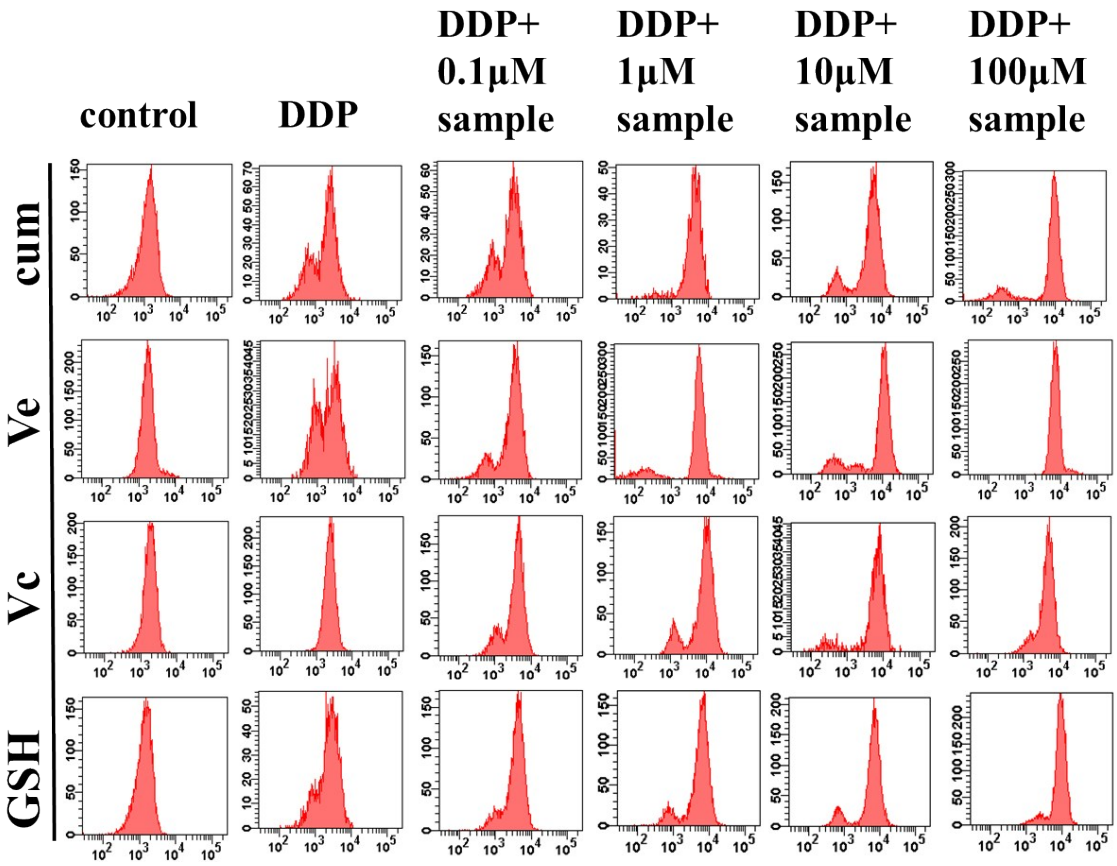


Fig. S7. Intracellular ROS detection using a commercialized ROS probe 2,7-dichlorodihydrofluorescein diacetate (λ_{ex} = 488 nm, λ_{em} = 500-530 nm) in Fig.4.

10. Cell damage analysis in Fig.4

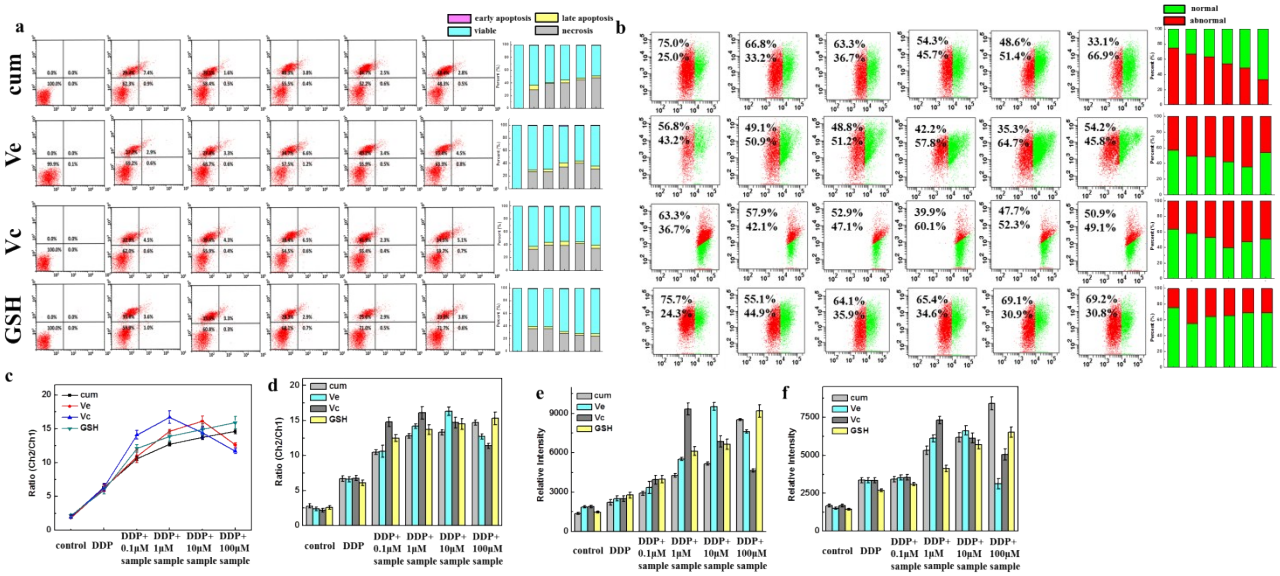


Fig. S8. (a) Apoptosis analysis by Annexin V/7-AAD. (b) ΔΨm analyzed by JC-1. (c) Average ratio intensities analysis for Fig.4. (d) Average ratio intensities analysis using flow cytometry analysis in Fig.4. (e) Relative intensities analysis for Fig.S7. (f) Relative intensities analysis for Fig.S6. The data were shown as mean (±s.d.) (n = 7).

11. Bright-Field Images of Fig. 2a.

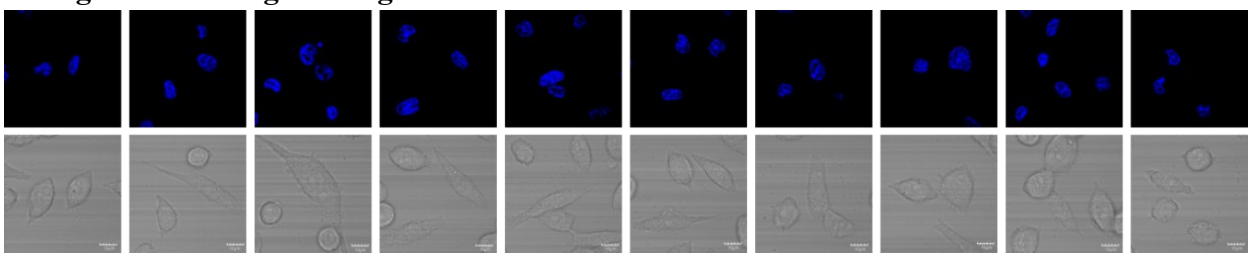


Fig. S9. Bright-Field Images of Fig. 2a

12. Bright-Field Images of Fig. 4

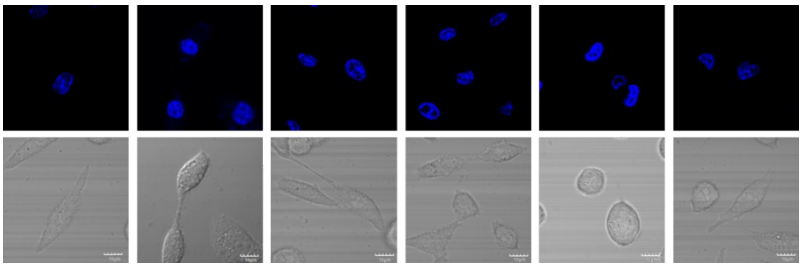


Fig. S10. Bright-Field Images of Fig. 4.

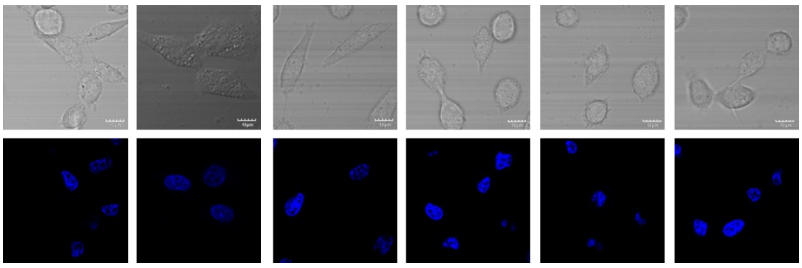


Fig. S11. Bright-Field Images of Fig. 4.

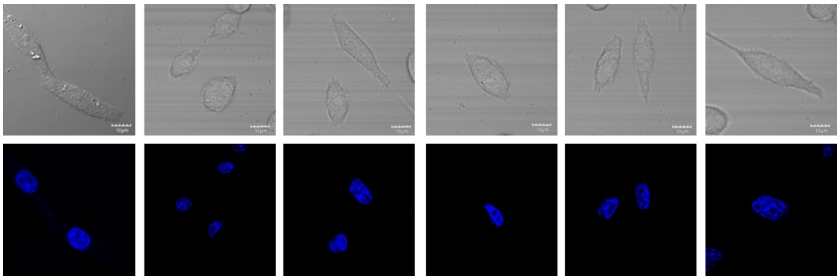


Fig. S12. Bright-Field Images of Fig. 4.

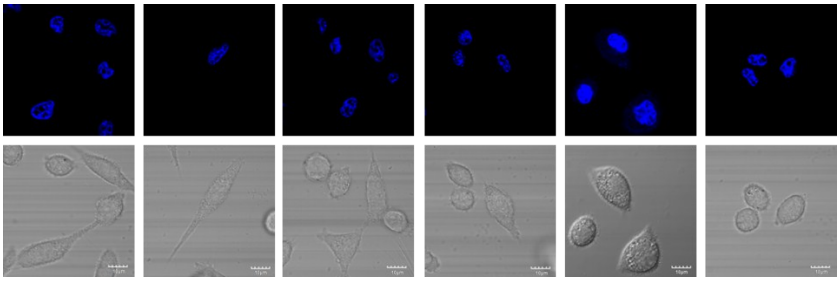


Fig. S13. Bright-Field Images of Fig. 4.

13. Bright-Field Images of Fig. 5

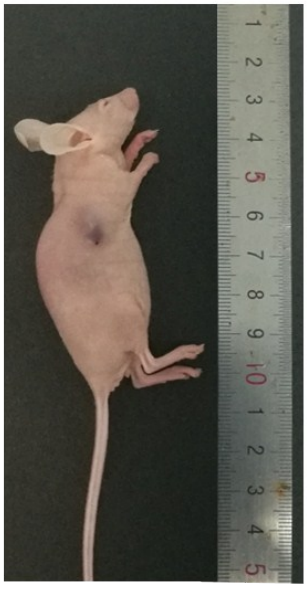


Fig. S14. Bright-Field Images of Fig. 5.

14. The comparison of this work with other two-photon fluorescent probes

We compared TP-Tfs with other two-photon fluorescent probes from linear range, detection limit to two-photon action cross-section in Table S1, and the results demonstrated that TP-Tfs showed good spectral characteristics.

Table S1

Probe	Detection species	Linear range	Detection limit	Two-photon action cross-section	Reference
TP-Tfs	$O_2^{\bullet-}$	0 - 20 μM	37 nM	126 GM	This work
Probe 1,	$O_2^{\bullet-}$	0 - 0.5 μM	1 nM	72 GM	16
HQ	$O_2^{\bullet-}$	0-500 μM	None	40 GM	17
ER-BZT	$O_2^{\bullet-}$	None	60 nM	65 GM	18
MF-DBZH	$O_2^{\bullet-}$	2 - 12 μM	9.5 nM	None	19
PY-CA	$O_2^{\bullet-}$	0 - 10 μM	3.2 nM	None	20
NpRbH	$O_2^{\bullet-}$	None	95.1 nM	None	21
NS-O	$O_2^{\bullet-}$	None	171 nM	None	22
TFR-O	$O_2^{\bullet-}$	0 - 6 μM	82 nM	None	23
CST	$O_2^{\bullet-}$	0 - 10 μM	0.37 nM	None	24
Mito-TP-ClO	hypochlorite	0 - 10 μM	25 nM	267 GM	25
1-Red	hydroxyl radicals	0 - 80 μM	None	25 GM	26
SPS-M1	hydrogen polysulfide	10 - 50 μM	0.1 μM	108 GM	27

15. Reference

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