Supporting Information

CONTENTS

1. Materials and methods

1.1 Reagents and instruments

The recombinant rat TrxR1, with a specific activity of 50% of the wild TrxR1 with the DTNB assay, was a gift from Prof. Arne Holmgren at Karolinska Institute, Sweden. NADPH was obtained from Roche (Mannheim, Germany). Auranofin (AF), Dulbecco's modified Eagle's medium (DMEM), glutathione (GSH), esterase, cysteine (Cys), yeast glutathione reductase (GR) and dimethyl sulfoxide (DMSO)were obtained from Sigma-Aldrich (St. Louis, USA). The HeLa cells and HepG2 cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All organic solvents and starting materials for organic synthesis are analytically pure and purchased from commercial supply. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Advance 400 or Varian 400, and tetramethylsilane (TMS) was used as a reference. MS spectra were recorded on Bruker Daltonics esquire 6000 mass spectrometer and Shimadzu LCMS-2020. HPLC was recorder on Shimadzu LCMS-2020 system with a Wondasil C18 Superb reversed-phase column (5 mm, 4.6 × 150 mm). The fluorescence images of living cells, zebrafish, and brain of mice were taken with a fluorescence microscope (Olympus FV1000 microscope). All procedures for *in vivo* imaging were carried out in accordance with the institutional guidelines (Guidance of the Care and Use of Laboratory Animals) and all *in vivo* experiments were approved by the Ethics Committee of Lanzhou University, China.

1.2 Cell culture and cytotoxic activity assay

HeLa cells were seeded in a 6-well plate and added DMEM supplemented with 10% PBS, 2 mM glutamine, penicillin (100 units/mL), and streptomycin (100 units/mL) at 37°C in a humidified environment of 5% CO2 overnight. Cells were incubated with probe TP-TRFS (0, 2.5, 5, 10, 20 and 40 μM) in triplicate in a 96-well plate for 24 h at 37 °C.

1.3 Stroke model

C57BL/6 mice were used in this study, they were obtained from Jackson Laboratory and fed in animal facilities at School of Basic Medical Sciences of Lanzhou University. All animal experiments have been approved by the university's Ethics Committee.

In this research, bilateral common carotid artery ligation (BCAL) ischemic model was used to induce the global ischemia upon mice cerebrum. Procedures were following several preliminary studies 12. Briefly, mice were anesthetized with a mixed solution of ketamine hydrochloride (150 mg/kg body weight) and xylazine (25mg/kg body

weight) then positioned gently on a heating pad to maintain the body temperature at 37℃ ± 0.5℃. After making an incision alone the scalp over skull, a custom-made metal frame was adhered onto the skull with Superglue and then fixed tightly to a steel plate. Subsequently, an area with the diameter of 2 mm was thinned to 20 μm on the skull using high speed dental drill, aiming to observe the blood flow of vessels within dura. Then, another incision was made alone the neck, bilateral common carotid arteries were carefully separated from carotid sheaths and encircled with surgical sutures. To induce ischemia, those surgical sutures were tied tightly, and mouse was softly moved under a stereomicroscope. If the decrease and stasis of blood flow could be observed, the ischemic model would be considered as successful. Mice were subjected to 60-munite ischemia, afterwards, sutures were loosened to induce reperfusion. Note that during BCAL, the animals should be moved as less as possible to reduce the mortality. [1-4]

1.4 Spectrophotometric Experiments.

Unless otherwise stated, all the enzymatic reactions were performed at 37 oC in TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.4). The excitation and emission slit widths are both 5 nm. For fluorescence measurements, compounds were dissolved in DMSO or distilled water to obtain stock solutions (1-10 mM), and diluted to desired concentrations by TE buffer.

1.5 Reduction of TP-TRFS by Recombinant TrxR and Cell Lysates.

TP-TRFS (10 μM), different concentrations of TrxR, and NADPH (200 μM) were mixed in TE buffer and incubated at 37 °C. Fluorescence spectra were recorded at specified time points. The lysates were prepared from Hep G2 cells by lysing them with RIPA buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.5% deoxycholate, 150 mM NaCl, 1% TritonX-100, 0.1% SDS, 1 mM Na3VO4, and 1 mM PMSF). Using BSA as the standard, the total protein concentrations were quantified by the Bradford procedure. To determine the ability of Hep G2 cell lysates to reduce TP-TRFS, additional NADPH (200 μM) was provided. For the DNCB and AF inhibition assays, the lysates were pre-reduced with NADPH (200 μM) for 15 minutes, and then incubated with DNCB (20 μM) or AF (2 μM) for another 30 minutes at room temperature.

1.6 Two-photon Fluorescence Imaging of TrxR in Living Cells.

HeLa, HeLa-shNT, HeLa-shTrxR1 and Hep G2 cells were seeded in 6-well plates and maintained in DMEM medium supplemented with 10% PBS, 2 mM glutamine, penicillin (100 units/mL), and streptomycin (100 units/mL) at 37 °C in a humidified environment of 5% CO_2. The cells were allowed to grow overnight. TP-TRFS (10 μM) was added and incubated for another 4 h. In order to inhibit TrxR activity in cells, the cells were treated with AF (2 μM) for 1.5 h before TP-TRFS was loaded. All cells were washed three times with PBS before two-photon imaging. The fluorescence images were acquired by a two-photon fluorescence microscope (Olympus FV1000).

1.7 Two-photon Fluorescence Imaging of TrxR in Zebrafish.

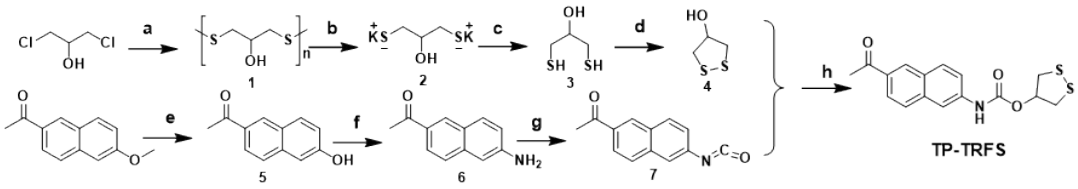
The 6-day-old zebrafish were collected and washed with standard zebrafish E3 medium, and then incubated with TP-TRFS (2 μM) in E3 medium at 28 °C for 2 h, as described in our recent publications.[5, 6] The zebrafishes were washed three times with E3 medium before two-photon imaging to remove the remaining TP-TRFS. For two-photon imaging, the zebrafishes were anesthetized with tricaine and implanted in 0.5%

agarose gel according to the literature methods.[7] The fluorescence images were acquired by a two-photon fluorescence microscope (Olympus FV1000 microscope).

1.8 Two-photon Fluorescence Imaging of TrxR activity in the Brains of Mice with Cerebral Ischemia Reperfusion Injury.

The stroke model was referred to the literature from ours and others.[1, 3, 4] To incubate the cerebral tissue with TP-TRFS, a cranial window above mouse skull was made. The edge of the same area used as observation window on the mouse skull was carefully thinned with the dental drill, and then a bone flap was removed softly with curved forceps after the bone was thinnedadequately. Sterilized artificial cortical-spinal solution (ACSF) was occasionally dropped on the exposed brain tissue to avoid it from drying. During surgery, any leaked blood was soaked and cleaned by a piece of moistened gelfoam. TP-TRFS (1 mM, 50 μL) was administered on the brain tissue and a cover glass (d= 3 mm) was then attached to the skull and sealed with dental cement. The incubation was allowed to last for 2 h, during which period the body temperature of the animal was kept by a heating pad.[1, 3] The mouse was firstly subjected to 60-minute global cerebral ischemia, then incubated with TP-TRFS for 2 h. After the incubation has been well achieved, the mouse was moved gently under a 25x water-immersion objective (Olympus FV1000) to be subjected to intravital imaging. The cortical region was imaged through cranial window repeatedly and 1024*1024 image stacks were collected with a step size of 1 μm. Note that the cortex should be exposed to the laser as less as possible to avoid possible tissue damages.

2. Experiment results



Regents and conditions: (a) S_8, N_2H_4, H_2O, $NH_2CH_2CH_2OH$; (b) N_2H_4, H_2O, KOH; (c) HCLl, 0 °C, air; (d) $NaHCO_3$, I_2, DCM, $Na_2S_2O_3$; (e) HCl, reflux; (f) NH_3,150 °C; (j) triphosgene, DMAP, toluene, reflux; (h) TEA, DCM.

Scheme S1. Synthesis of TP-TRFS

2.1 Synthesis of TP-TRFS.

For the synthesis of TP-TRFS, to a solution of compound 6 (74 mg, 0.4 mmol) and triphosgene (240 mg, 0.8 mmol) in dry toluene (15 mL) was added DMAP (147 mg, 1.2 mmol), and the solution was refluxed for 4 h. The solution was evaporated under reduced pressure to give compound 7. Then the compound 7 was redissolved in triethylamine (101 mg, 1.0 mmol)/DCM (15 mL), to this solution was added compound 4 (122 mg, 1.0 mmol) in anhydrous CH_2Cl_2 (15 mL) dropwise at 0 °C. After that, the reaction mixture was stirred at room temperature overnight, and then concentrated under reduced pressure to obtain the solid residue, which was further purified by silica gel chromatography with petroleum ether/ethyl acetate (10:1) to obtain a white solid TP-TRFS (66 mg, 50% yield). ^{1}H NMR (400 MHz,$CDCl_3$) δ 8.42 – 8.39 (s, 1H), 8.06 – 8.00 (m, 2H), 7.92 (d, J = 8.8 Hz, 1H), 7.82 (d, J = 8.7 Hz, 1H), 7.44 (dd, J = 8.8, 2.2 Hz, 1H), 6.97 (s, 1H), 5.86 (m, 1H), 3.47 – 3.32 (m, 4H), 2.71 (s, 3H). ^{13}C NMR (100

MHz, DMSO-d_6) δ 197.97, 153.16, 139.54, 136.33, 133.16, 130.91, 130.62, 128.79, 127.91, 124.60, 120.56, 113.83, 78.77, 45.11, 27.07. HRMS (m/z): $[M-H]^-$ calcd for 332.0421, found 332.0420

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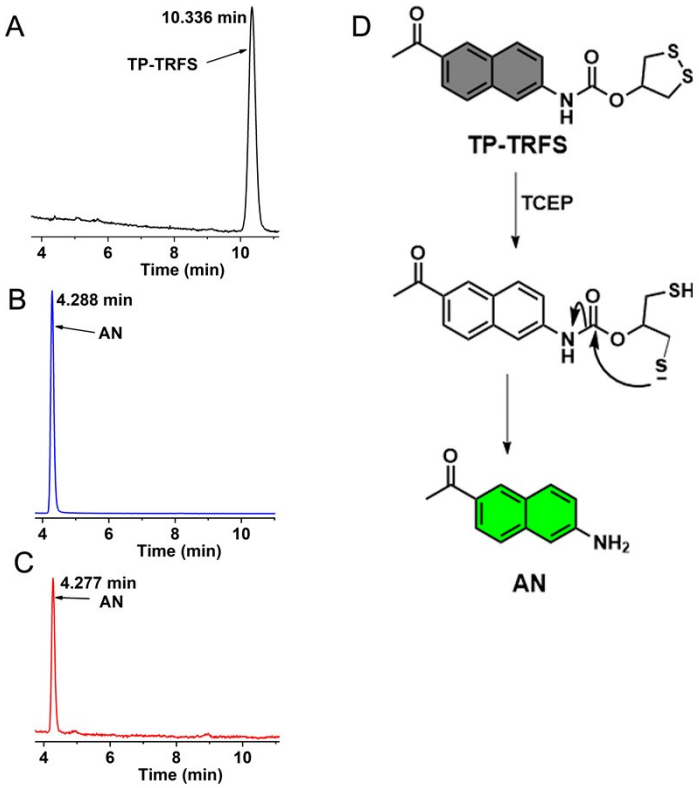

Figure S1. HPLC analysis of the reaction of TP-TRFS with TCEP. (A) TP-TRFS (20 μM) (330 nm); (B) AN (20 μM) (370 nm) as standard samples; (C) TP-TRFS (20 μM) incubated with TCEP (1 mM) at 37 °C for 3 h in TE buffer (370 nm); (D) Recognition Mechanism for TP-TRFS.

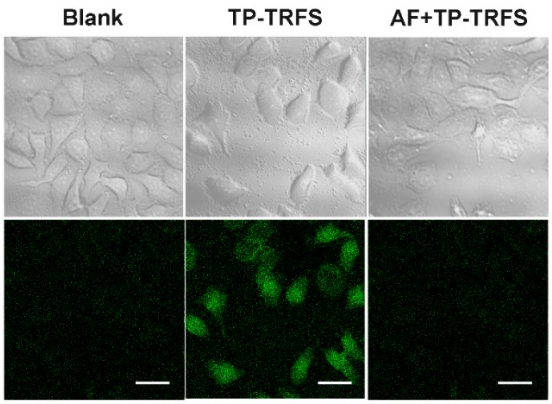

Figure S2. Fluorescence changes of TP-TRFS in living Hep G2 cells. (a) Hep G2 cells only (left), Hep G2 treated with TP-TRFS (10 μM) for 4 h (middle), and Hep G2 cells treated with AF (2 μM) for 1.5 h followed by further treated with TP-TRFS (10 μM) for 4 h. Scale bar: 20 μm.

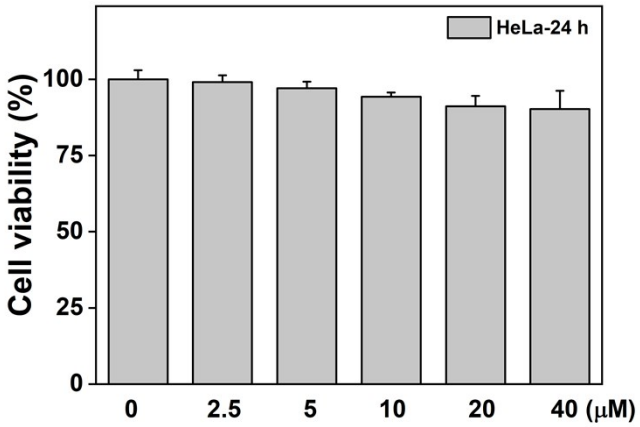

Figure S3. Cell viability of HeLa cells at various concentrations of TP-TRFS using MTT assay.

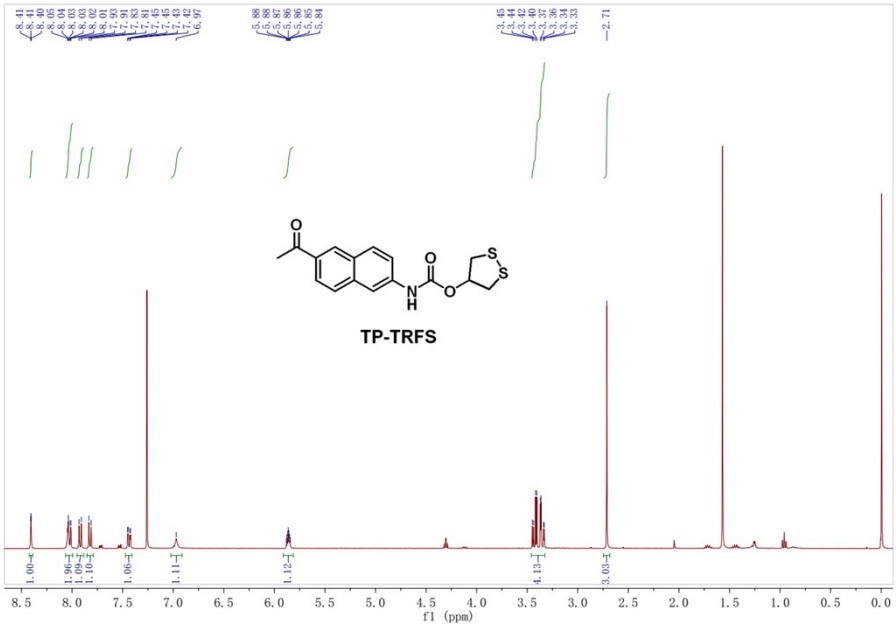

Figure S4. ^{1}H NMR spectral of compound TP-TRFS in $CDCl_3$

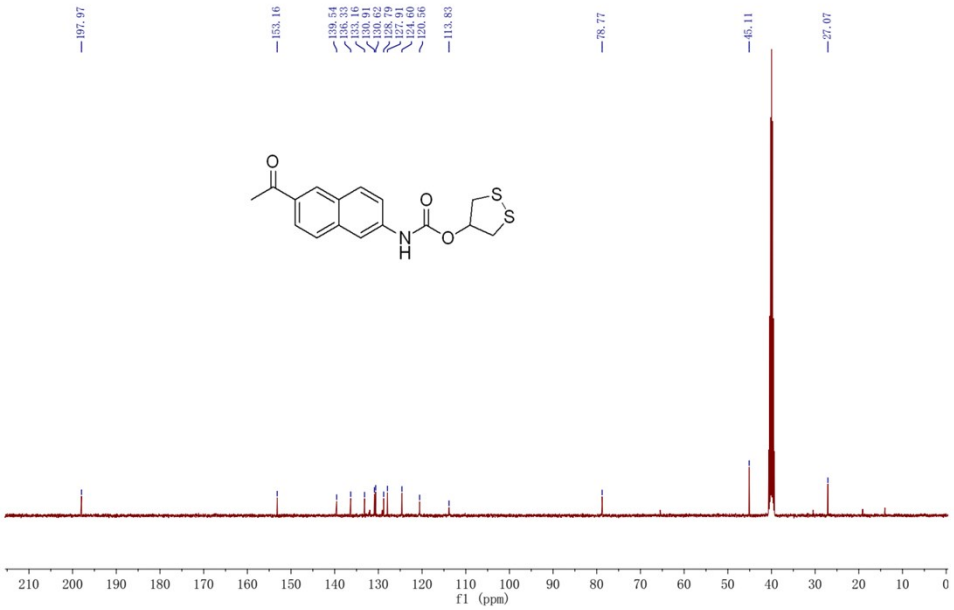

Figure S5. ^{13}C NMR spectral of compound TP-TRFS in $CDCl_3$

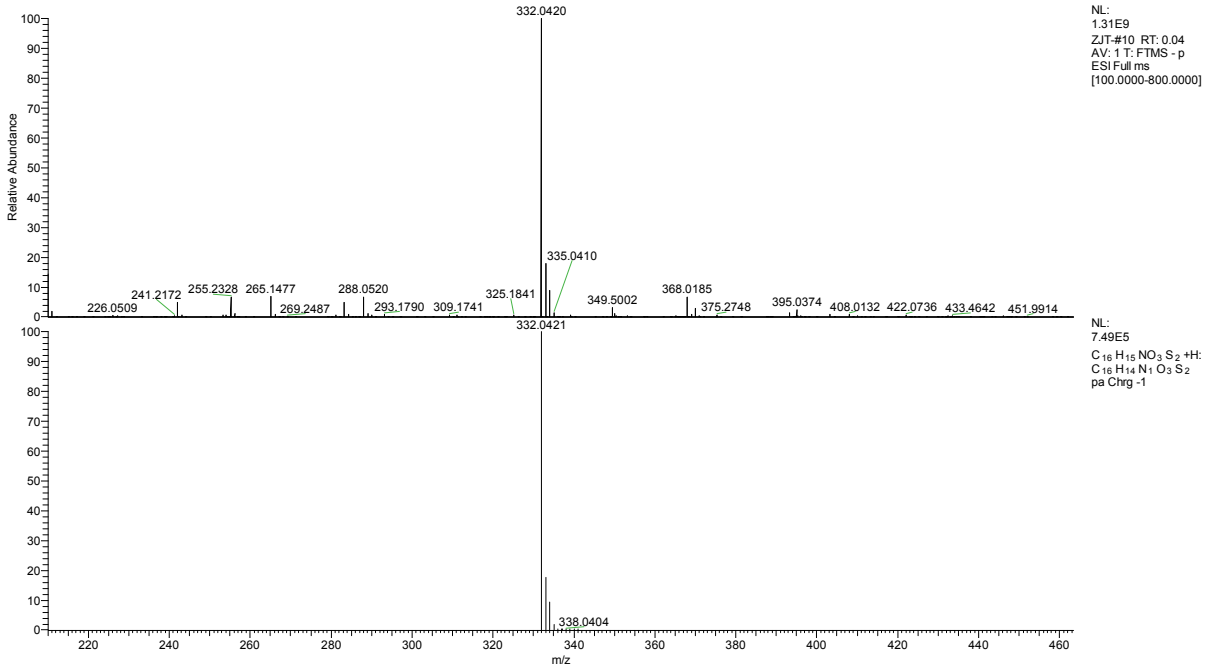

Figure S6. High resolution mass spectral of compound TP-TRFS

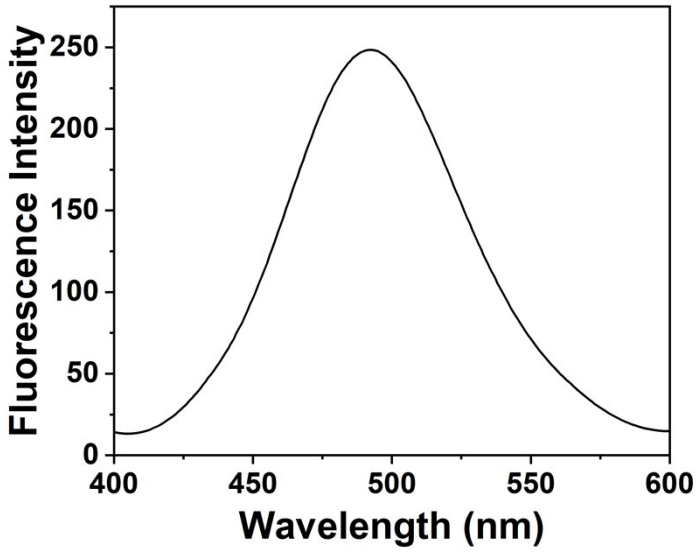

Figure S7. The fluorescence spectra of compound AN (20 μM, TE buffer) (excited at 370 nm).

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