SUPPORTING INFORMATION

Fast response and red emission probe for mammalian thioredoxin reductase

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EXPERIMENTAL SECTION

Materials
The recombinant rat TrxR was essentially prepared as described\(^1\) and is a gift from Prof. Arne Holmgren at Karolinska Institute, Sweden. The recombinant U498C TrxR mutant (Sec→Cys) was produced as described.\(^2,3\) The HeLa cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Dulbecco’s modified Eagle’s medium (DMEM), reduced glutathione (GSH), dimethyl sulfoxide (DMSO), glutathione reductase (GR) were obtained from Sigma-Aldrich (St. Louis, MO, USA). NADPH was obtained from Roche (Mannheim, Germany). Ethylene diaminetetraacetic acid (EDTA) was obtained from J&K Scientific (Beijing, China). Fetal bovine serum (FBS) was obtained from Sijiqing (Hangzhou, China). Penicillin and streptomycin were obtained from Sangon (Shanghai, China). Acylase from Hog Kidney and L-selenocystine were obtained from J&K Scientific (Beijing, China). The Sec (10 \(\mu\)M) was generated in situ by mixing Cys (1 mM) and selenocytine (5 \(\mu\)M). All other reagents were of analytical grade and were purchased from commercial supplies. Absorption spectra were recorded on UV–vis spectrometer evolution 200 (Thermo Scientific). Fluorescence studies were carried out using a Cary Eclipse Fluorescence Spectrophotometer (Agilent) or Floid cell imaging station. MS spectra were recorded on Trace DSQ GC-MS spectrometer or Bruker Daltonics esquire 6000 mass spectrometer. HRMS was obtained on Orbitrap Elite (Thermo Scientific). The quantum yields (\(\phi\)) of 5 and TRFS-red were determined on FLS920 spectrometer (Edinburgh Instruments, U.K.). Melting points (mp) were determined on a Fisher-Johns melting apparatus and were uncorrected. \(^1\)H and \(^{13}\)C NMR spectra were recorded on Bruker Advance 400, and tetramethylsilane (TMS) was used as a reference. The organic solvent (DMSO) is no more than 1 % (v/v) in the in vitro assays, and no more than 0.1 % (v/v) in the cell experiments.

Chemical synthesis
Compounds 1 and 2 were prepared by adapting the published procedures.\(^4,5\)

Synthesis of compound 3\(^6\)
Under an argon atmosphere, a mixture of m-aminophenol (2.73 g, 25 mmol), methyl acrylate (13.5 g,
156 mmol), sodium bromide (625 mg, 6 mmol) and 3.1 mL acetic acid was stirred at 95°C for 19 h. After 19 h, the mixture was diluted by 150 mL water, neutralized by sodium bicarbonate and abstracted by ethyl acetate. The organic layer was dried by Na₂SO₄, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, 2:1 petroleum ether/ethyl acetate) to afford compound 3 as a yellow semisolid (6.05 g, 86%). ¹H NMR(400 MHz, CDCl₃) δ: 2.58-2.62 (t, 4H, J = 6.8 Hz), 3.61-3.65 (t, J = 6.8 Hz, 4H), 3.69 (s, 6H), 5.20 (s, 1H), 6.20-6.23 (m, 2H), 6.27-6.28 (m, 1H), 7.05-7.10 (m, 1H); ¹³C NMR (100 MHz, CDCl₃) δ: 32.3, 46.9, 51.8, 99.6, 104.1, 105.2, 130.4, 148.3, 157.0, 172.6; ESI-MS (m/z): [M+H]⁺ 282.0.

Synthesis of compound 4′
A solution of 4-nitroaniline (1.1 g, 8 mmol) was dissolved in a mixture of 5 mL of concentrated hydrochloric acid and 5 mL of water. The reaction flask was cooled in an ice-bath and sodium nitrite (550 mg, 8 mmol) dissolved in 5 mL of water was added slowly. The reaction mixture was stirred at 0°C for an additional 20 min. At the end of the reaction, the solution turned orange. Then a solution of compound 3 (1.97 g, 7 mmol) in 2 mL of methanol was added to the reaction flask. The reaction mixture was stirred for 30 min at room temperature. The red precipitate was filtered and washed with cold ethanol. The crude product was recrystallized in ethanol. After drying, compound 4 (2.26 g, 75%) was obtained as an orange red solid. ¹H NMR (400 MHz, CDCl₃) δ: 2.68-2.71 (t, J = 7.2 Hz, 4H), 3.72 (s, 6H), 3.78-3.82 (t, J = 7.2 Hz, 3H), 6.03 (d, J=2.4 Hz, 1H), 6.46-6.49 (dd, J₁=2.4 Hz, J₂ = 9.2 Hz, 1H), 7.51-7.53 (d, J = 9.2 Hz, 1H), 7.77-7.99 (d, J = 8.8 Hz, 2H), 8.29-8.31 (d, J = 9.2 Hz, 2H), 14.83 (s, 1H); ¹³C NMR (100 MHz, CDCl₃) δ: 32.4, 47.0, 52.0, 98.7, 108.1, 120.0, 125.1, 132.7, 135.93, 146.1, 152.8, 153.2, 162.6, 171.6; mp: 128.4-128.9°C; ESI-MS (m/z): [M+H]⁺ 431.4.

Synthesis of compound 5′
A mixture of compound 4 (731 mg, 1.7 mmol), 1-aminonaphthalene (272 mg, 1.9 mmol) and 30 mL of DMF containing perchloric acid (1 mL, 70%) was heated to 155-160 °C for 15 min with stirring. The color of the reaction mixture changed from brown to deep blue. The reaction was monitored by silica gel TLC. After cooling, the DMF was evaporated to dryness under reduced pressure. The crude perchlorate salt was purified by flash column chromatography (silica gel, 1:40 methanol/dichloromethane) to afford compound 5 as a blue solid (617 mg, 68%). ¹H NMR (400 MHz, DMSO-d₆) δ: 2.72-2.76 (t, J = 7.2 Hz, 4H), 3.64 (s, 6H), 3.86-3.90 (t, J = 7.2 Hz, 4H), 6.87 (s, 1H), 7.09-8.00 (d, J₁=2.4 Hz, 1H), 7.25-7.28 (dd, J₁=2.4 Hz, J₂=9.2 Hz, 1H), 7.88-7.45 (dd, J₁=9.2, J₂=17.2 Hz, 2H), 8.01-8.04 (t, J = 7.6 Hz, 1H), 8.48-8.50 (d, J = 8.0 Hz, 1H), 8.82-8.84 (d, J = 8 Hz, 1H), 10.05-10.12 (d, J = 19.2 Hz, 2H); ¹³C NMR (100 MHz, DMSO-d₆) δ: 31.4, 46.7, 51.6, 96.7, 97.0, 114.6, 124.0, 124.3, 128.7, 130.2, 131.4, 132.3, 132.9, 135.4, 147.4, 151.6, 153.2, 161.8, 171.5; mp: 202.7-203.2°C; ESI-MS (m/z): [M⁺] 434.4.

Synthesis of compound TRFS-red₈,₉
Compound 2 (122 mg, 1 mmol) was dissolved in anhydrous DCM (10 mL), and DIPEA (776 mg, 6
mmol) was added. Then a solution of triphosgene (1.19 g, 4 mmol) in anhydrous DCM (10 mL) was added dropwise at 0°C and stirred for 2 h. After cooling to room temperature, the reaction mixture was flushed with nitrogen gas to remove the unreacted phosgene gas (CAUTION: TOXIC) and neutralization in an NaOH bath, the mixture was added dropwise to the solution of compound 5 (150 mg, 0.28 mmol) and TEA (132 mg, 1.3 mmol) in dry THF/DCM (1:1, v/v, 10 mL) at 0°C. The reaction mixture was stirred overnight at room temperature. The solvent was evaporated and the residue was purified by column chromatography (silica gel, 1:100 methanol/dichloromethane) to afford TRFS-red as a purple red solid (53 mg, 28%).

\[\text{\textsuperscript{1}H NMR (400 MHz, CDCl}_3) \delta: 2.65-2.69 (t, J = 7.2 Hz, 4H), 3.40-3.44 (dd, J_1 = 3.2 Hz, J_2 = 12.8 Hz, 2H), 3.51-3.55 (dd, J_1 = 5.6 Hz, J_2 = 12.8 Hz, 2H), 3.73 (s, 6H), 3.75-3.78 (t, J = 7.2 Hz, 4H), 5.80-5.85 (m, 1H), 6.44-6.45 (d, J = 2.4 Hz, 1H), 6.62 (s, 1H), 6.65-6.68 (m, 1H), 7.59-7.64 (m, 2H), 7.68-7.71 (t, J = 7.2 Hz, 1H), 8.43-8.45 (d, J = 7.6 Hz, 1H), 8.62-8.64 (d, J = 8 Hz, 1H); \text{\textsuperscript{13}C NMR (100 MHz, CDCl}_3) \delta: 32.1, 45.0, 47.0, 52.0, 79.9, 97.2, 100.3, 110.0, 124.0, 125.6, 125.9, 130.3, 131.1, 131.2, 131.3, 131.5, 141.5, 146.3, 149.4, 149.7, 161.7, 161.2, 171.8; mp: 160.9-161.1°C; ESI-MS (m/z): [M]+ 582.5; HRMS (ESI) calculated for [C_{28}H_{28}N_{3}O_{7}S_{2}]^+ [M]+ requires m/z = 582.1363, found 582.1357.

**UV–vis and Fluorescence Spectroscopy**

UV–vis spectra were acquired from UV–vis spectrometer evolution 200 (Thermo Scientific). Fluorescence spectroscopic studies were performed with Cary Eclipse Fluorescence Spectrophotometer (Agilent). The slit width was 5 nm for both excitation and emission. For spectra measurements, TRFS-red was dissolved in DMSO to obtain a stock solution, which was diluted with TE to the desired concentrations.

**Live Cell Imaging**

HeLa cells were seeded in a 12-well plate and cultured overnight in DMEM supplemented with 10 % FBS, 2 mM glutamine, penicillin (100 units/mL), streptomycin (100 units/mL) at 37 °C in a humidified atmosphere of 5 % CO₂. a) TRFS-red (1 μM) was added, and the cells were incubated for another 0, 30, 60 and 120 min respectively. The cells were rinsed with the medium for three times to remove the remaining TRFS-red, then the fluorescence images were acquired with Floid cell imaging station and the relative fluorescence intensity of single cell in images was quantified by the software of ImageJ. b) To inhibit the cellular TrxR, the cells were treated with auranofin (2 μM) or DNCB (20 μM) for 3 h and then the cells were treated with TRFS-red (1 μM) for 1 h. The cells were rinsed with the medium for three times to remove the remaining TRFS-red, then the fluorescence images were acquired with Floid cell imaging station and the relative fluorescence intensity of individual cells in images was quantified by the software of ImageJ. c) TRFS-red (1 or 10 μM) or TRFS-green (1 or 10 μM) was added, and the cells were incubated for another 1, 2 and 4 h respectively. The cells were rinsed with the medium for three times to remove the remaining TRFS-red or TRFS-green, then the fluorescence images were acquired with Floid cell imaging station.

**HPLC Analyses of the Reaction between TRFS-red and TCEP**
TRFS-red (10 μM) was incubated with TCEP (1 mM) in an acetonitrile-H\textsubscript{2}O mixed solvent (1:40, v/v) at 37°C for 3 h. All samples were passed through a 0.22 μm filter, and 100 μL of sample was loaded onto the Waters symmetry C18, reversed-phase column (3.5 μm, 4.6 × 75 mm) on a Waters 1525 binary HPLC system. The mixture of acetonitrile and water (13:7, v/v) was used as eluent at the flow rate of 1.0 mL min\textsuperscript{-1}. The detection wavelength for TRFS-red and compound 5 was set at 268 nm.

**Reference**

**Figure S1** (A) Time course of absorbance change of TRFS-red (10 μM) in the presence of TCEP (1 mM) at 37°C. Arrows in (A) show the change of absorbance at 530 nm and 615 nm. (B) Time course of fluorescence change of TRFS-red (10 μM) in the presence of TCEP (1 mM) at 37°C. The arrow in (B) shows the change of emission at 661 nm excited at 615 nm. (C) Absorbance (C) and emission (D) spectra of TRFS-red, TRFS-red + TCEP (2h) and the fluorophore 5 ($\lambda_{ex} = 615$ nm).
Figure S2 Determination of $K_m$ and $k_{cat}$ values of TRFS-green (left) and TRFS-red (right).

Figure S3 Imaging cytosolic TrxR activity by TRFS-red. HeLa cells were treated with varying concentrations of TRFS-green for 1 h (red channel), and then were further stained with Hoechst 33342 (5 $\mu$g/ml, blue channel) for 10 min. Scale bar: 20 $\mu$m.
**Figure S4** HPLC analyses of the reaction product of TRFS-red. (A) Compound 5 (10 µM) (268.5 nm), and (B) compound TRFS-red (20 µM) (268.5 nm) as standard samples; (C) TRFS-red (10 µM) incubated with TCEP (1 mM) at 37°C for 3h (268.5 nm). All samples were passed through a 0.22 µm filter, and 100 µL of sample was loaded onto Waters-symmetry C18, reversed-phase column (3.5 µm, 4.6×75 mm) on a Waters 1525 binary HPLC system. The column was eluted with acetonitrile/water (13:7, v/v). The flow rate was set at 1.0 mL min⁻¹. A UV detector was used to monitor the desiring product at wavelength from 200-400 nm.

| Table S1 HPLC analysis of the conversion of TRFS-red reduced by TCEP. |
|-----------------|-----------------|
| **TRFS-red**    | **Compound 5**  |
| Retention time: 16.43 min | Retention time: 6.72 min |
| Before reaction | After 3 h       | Before reaction | After 3 h       |
| 10 µM           | 1.44 µM         | 0 µM           | 8.51 µM         |
Figure S5 $^1$H NMR Spectrum of compound 3 in CDCl$_3$ (400 MHz).

Figure S6 $^{13}$C NMR Spectrum of compound 3 in CDCl$_3$ (100 MHz).
Figure S7 ESI-Mass spectrum of compound 3 (ESI-MS).
Figure S8 $^1$H NMR Spectrum of compound 4 in CDCl$_3$ (400 MHz).

Figure S9 $^{13}$C NMR Spectrum of compound 4 in CDCl$_3$ (100 MHz).
**Figure S10** ESI-Mass spectrum of compound 4 (ESI-MS).
Figure S11 $^1$H NMR Spectrum of compound 5 in CDCl$_3$ (400 MHz).

Figure S12 $^{13}$C NMR Spectrum of compound 5 in CDCl$_3$ (100 MHz).
Figure S13 ESI-Mass spectrum of compound 5 (ESI-MS).
Figure S14 $^1$H NMR Spectrum of compound TRFS-red in CDCl$_3$ (400 MHz).

Figure S15 $^{13}$C NMR Spectrum of compound TRFS-red in CDCl$_3$ (100 MHz).
Figure S16 ESI-Mass spectrum of compound TRFS-red (ESI-MS).
Figure S17 HR-Mass spectrum of compound TRFS-red (ESI).