Supporting Information

Depletion of protein thiols and accumulation of oxidized thioredoxin in Parkinsonism disclosed by a red-emitting and environment-sensitive probe

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References
**Materials and instruments.** Chemical reagents and solvents were purchased from commercial sources and used as received without further purification. $^1$H NMR and $^{13}$C NMR spectra were measured on a Bruker AVANCE III 400 NMR spectrometer using CDCl$_3$ as solvents. HRMS spectra were recorded on Bruker Daltonics mass spectrometer. The cell fluorescence imaging was performed with a Floid cell imaging station microscope and the gel fluorescence imaging was performed by an imageQuant LAS 400 (GE Healthcare). Proteins were analyzed by MALDI-TOF-MS on a Bruker autoflex speed mass spectrometer. 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.

**Scheme S1 Synthesis of FM-red**

![Scheme S1 Synthesis of FM-red](image)

*Synthesis of FM-red, Reagents and conditions: (a) AcOH, reflux for 2 h, 44%; (b) concentrated H$_2$SO$_4$, 90°C, 1.5 h, 80%; (c) 4-(N-Boc-amino)piperidine, EDC, HOBT, CH$_2$Cl$_2$, RT, 6 h, 75%. (d) i) CF$_3$COOH, CH$_2$Cl$_2$, RT, overnight; ii) 1, EDC, HOBT, CH$_2$Cl$_2$, RT, 6 h, 55%.*
Compounds 1 - 3 were synthesized according to the reference.\(^1,2\)

**Synthesis of compound FM-red:** Compound 3 (0.62 g, 1.0 mmol) was dissolved in anhydrous dichloromethane (20 mL) in ice bath, and then anhydrous CF\(_3\)COOH (10 mL) was slowly added. The solution was stirred overnight at room temperature. The mixture solution was respectively washed with saturated NaHCO\(_3\) solution three times, saturated salt solution three times and collected organic phase. The solvent was then evaporated under reduced pressure and the resulting residue was mixed with 1 (0.22 g, 1.2 mmol), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (0.47 g, 2.0 mmol), and 1-Hydroxybenzotriazole (0.27 g, 2.0 mmol) in dichloromethane (30 mL) was stirred overnight at room temperature and the solvent was removed under reduced pressure. The resulting crude product was purified by silica gel column chromatography to give a dark purple solid (0.37 g, 55%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\): 8.15-8.13 (d, \(J = 8\) Hz, 2H), 7.65-7.57 (m, 2H), 7.54-7.52 (d, \(J = 8\) Hz, 1H), 7.47-7.44 (m, 3H), 7.19 (s, 1H), 7.10-7.08 (d, \(J = 8\) Hz, 1H), 7.02-6.89 (m, 1H), 6.81-6.79 (d, \(J = 8\) Hz, 2H), 6.66 (s, 2H), 4.16-4.12 (d, \(J = 16\) Hz, 1H), 3.83 (s, 1H), 3.65-3.60 (m, 5H), 3.42-3.39 (t, \(J = 12\) Hz, 1H), 3.22-3.20 (s 1H), 3.08-2.94 (m, 2H), 2.32-2.28 (t, \(J = 16\) Hz, 1H), 2.17-2.15 (t, \(J = 8\) Hz, 2H), 1.85-1.80 (m, 4H), 1.35-1.31 (t, \(J = 16\) Hz, 6H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\): 172.09, 170.93, 167.57, 166.64, 158.15, 157.18, 155.31, 155.05, 154.56, 154.34, 136.03, 134.07, 131.22, 130.65, 129.85, 127.91, 115.93, 115.21, 114.95, 113.73, 112.87, 112.58, 108.59, 96.86, 45.83, 40.32, 37.08, 33.03, 31.59, 31.20, 30.95, 30.68, 29.62, 24.66, 12.53. HRMS (ESI): m/z calcd for C\(_{41}\)H\(_{46}\)N\(_5\)O\(_5\)\(^+\) [M]\(^+\) 688.3493; found: 688.3490.

**Spectrophotometric measurement.** Both the absorption spectra and fluorescence spectra were recorded in PBS (50 mM, pH 7.4). Stock solution of **FM-red** was prepared in DMSO and other
substances were in tri-distilled water. Samples for absorption and fluorescence measurements were contained in 1 cm × 1 cm quartz cuvettes (3 mL volume). Absorption spectra were recorded on UV–vis spectrometer evolution 200 (Thermo Scientific). Fluorescence studies were carried out using an Agilent Cary Eclipse fluorescence spectrophotometer at 37 °C. The slit width was 5 nm for both excitation and emission.

**Determination of Trx redox states by FM-red.**³ To a solution of 1.0 mg/mL Trx (200 μL) in PBS (50 mM, pH = 7.4, containing 1% SDS) was added 0.5 M TCEP (2 μL) or 0.5 M diamide (2 μL), and then the solution was incubated for 30 min at 37 °C. Then the cold acetone (1 mL) was added, and placed for 30 min at -20 °C. Samples were centrifugalized at 4 °C for 10 min and the supernatant was removed. Proteins were dissolved in PBS buffer (200 μL), and incubated with 0.5 M **FM-red** (4 μL) for 30 min at 37 °C. The solutions were boiled with loading buffer (4 ×) with dithiothreitol (DTT) for 5 min. Protein samples were analyzed by 18% SDS-PAGE and the gel was stained by coomassie brilliant blue (CBB).

**Western blotting detection of Trx redox states in HeLa cell lysate.**³ HeLa cell lysate was prepared by lysing the cells with PBS containing 1% SDS. The lysate was treated with 0.5 M TCEP (2 μL) or 0.5 M diamide (2 μL) for 30 min followed by acetone precipitation at -20 °C for 30 min to remove the reducing or oxidizine reagents. All samples were resuspended in PBS buffer containing 1% SDS (200 μL). 0.5 M **FM-red** (4 μL) or 0.5 M NEM (2 μL) was added to the solution and the solution was incubated for 30 min at 37 °C. The samples were then boiled with loading buffer (4 ×) with reducing agent (DTT) and separated by SDS-PAGE (18%). Proteins were transferred to PVDF
membranes, blocked with 5% nonfat milk at room temperature for 1 h, and incubated with the anti-human Trx1 antibody (Santa Cruz, 1:1000) overnight at 4°C. The membranes were washed three times with TBST solution and incubated with a horseradish peroxidase-conjugated secondary antibody (1:4000) at room temperature for 1 h. After washed three times with TBST solution, the target protein bands were detected by the enhanced chemiluminescence (GE healthcare).

**Western blotting detection of Trx redox states in PC12 cell lysate.**\(^4,5\) PC12 cells were pretreated with different concentrations of 6-OHDA (0, 50, 100, and 200 μM) for 8 h, and then incubated with FM-red (10 mM) for 30 min. PC12 cells were harvested and lysed with PBS containing 6 M GndHCl. The lysate was precipitated by acetone precipitation at -20 °C for 30 min to remove the excess probe. All samples were resuspended in PBS buffer (200 μL). The samples were then boiled with loading buffer (4 ×) with reducing agent (DTT) and separated by SDS-PAGE (18%). Proteins were transferred to the methanol-activated PVDF membranes (200 mA, 2 h), blocked with 5% nonfat milk at room temperature for 1 h, and incubated with the anti-human Trx1 antibody (Santa Cruz, 1:1000) overnight at 4°C. The membranes were washed three times with TBST solution and incubated with a horseradish peroxidase-conjugated secondary antibody (1:4000) at room temperature for 1 h. After washed three times with TBST solution, the target protein bands were detected by the enhanced chemiluminescence (GE healthcare).

**Mass Spectrometric Analysis.** The intact masses of modified and unmodified proteins were determined by MALDI-TOF-MS on a Bruker autoflex speed mass spectrometer. To a solution of 1.0 mg/mL recombinant *E.coli* Trx (200 μL) in PBS (50 mM, pH = 7.4) was added 0.5 M TCEP (2 μL),
and then the solution was incubated for 30 min at 37 °C. Then the cold acetone (1 mL) was added, and placed for 30 min at -20 °C. Proteins were collected after centrifugalized at 4°C for 10 min and the supernatant was removed. Proteins were dissolved in PBS buffer (200 µL), and incubated with 0.1 M **FM-red** (3 µL) for 30 min at 37 °C. Then the cold acetone (1 mL) was added, and placed for 30 min at -20 °C. Proteins were centrifugalized at 4°C for 10 min and the supernatant was removed. Proteins were dissolved in the mixture of acetonitrile distilled water (3:7, containing 0.5% trifluoroacetic acid). Sinapinic acid was used as matrix with 1:1 sample to matrix ratio.

**Cell culture and cytotoxic activity assay.** HeLa cells were cultured in DMEM (10% FBS, 2 mM glutamine, and 100 units/mL penicillin/streptomycin), and maintained in an atmosphere of 5% CO₂ at 37 °C. 1×10⁴ cells were incubated with **FM-red** in triplicate in a 96-well plate for the indicated time at 37 °C in a final volume of 100 µL. Cells treated with DMSO alone were used as controls. At the end of the treatment, 10 µL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C. An extraction buffer (100 µL, 10% SDS, 5% isobutanol, 0.1% HCl) was added, and the cells were incubated overnight at 37°C. The absorbance was measured at 570 nm on Multiskan GO (Thermo Scientific).

**Live cells fluorescence imaging.** HeLa cells were placed in 6-well plate, and then incubated with **FM-red** (10 µM) for 30 min at 37 °C. For blocking biothiols, the cells were pretreated with N-ethylmaleimide (NEM) (100 µM) for 30 min and then incubated with **FM-red** (10 µM) for 30 min at 37 °C. To reduce cellular GSH, the cells were pretreated with BSO (50 µM) for 12 h, after that, incubated with **FM-red** (10 µM) for 30 min at 37 °C. PC12 cells were pretreated with different
concentrations of 6-OHDA (0, 50, 100, and 150 μM) for 8 h, and then incubated with **FM-red** (10 mM) for 30 min. The cells were washed three times with PBS followed imaged under a Floid cell imaging station microscope.

**Zebrafishes fluorescence imaging**

The collected zebrafish embryos were washed using standard zebrafish E3 culture medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4), and then incubated with probe (10 μM) for 30 min at 28 °C in the E3 culture medium. For blocking biothiols, the zebrafishes were pretreated with N-ethylmaleimide (NEM) (50 μM) for 30 min and then incubated with **FM-red** (10 μM) for 30 min at 28 °C. The embryos were examined under a dissecting light microscope (Nanjing Jiangnan Novel Optics, China).
Fig. S1 (A) Absorption spectra of FM-red (10 μM) in different solvents at room temperature (25 °C). (B) Fluorescence spectra of FM-red (10 μM) in different solvents at room temperature (25 °C).

Fig. S2 (A) Fluorescence spectra of **FM-red** (10 μM) in 1, 4-dioane/water solvent with different fractions of 1, 4-dioane (f_d) at room temperature (25 °C). (B) Fluorescence spectra of **FM-red** (10 μM) in methanol/glycerol solvent with different fractions of glycerol (f_g) at room temperature (25 °C).
Fig. S3 Absorption spectra of free **FM-red** (10 μM) and after reacting with GSH (1
mM) (A), Cys (1 mM) (B) and Hcy (1 mM) (C) for 30 min at 37°C in PBS (50 mM, pH 7.4).

Fig. S4 Cell viability of HeLa cells at various concentrations of **FM-red** using MTT assay.
Fig. S5 $^1$H NMR spectra of compound **FM-red** in CDCl$_3$
Fig. S6 $^{13}$C NMR spectra of compound **FM-red** in CDCl$_3$
Fig. S7 HRMS spectra of compound **FM-red**.
References