Supporting information for

A cysteine-selective fluorescent probe for monitoring the stress response cysteine fluctuations
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1. Materials

The stock solution of the probe 1 was prepared in analytical DMSO and stored in refrigerator at 4 °C. Stock solutions of NaCl, KCl, CaCl$_2$, NaN$_3$, Hcy, Cys, GSH, L-Ascorbic acid, Tyr, Asp, His, Val, Pro, Phe, Ser, Gly, Thr were prepared in deionized water (10 mM, respectively). CCK-8 kit was obtained from Dojindo. NEM and TCEP was obtained from Aladdin. Annexin V-FITC Apoptosis Detection Kit, Beyotime. NucBlue Live cell Stain Ready Probes reagent (Hoechst 33342), Thermo Fisher Scientific. MitoTracker Deep Red FM, Thermo Fisher Scientific.

2. Instruments

Fluorescence spectra were obtained by a HITACHI F-4700 spectro fluorometer. Absorption spectra were collected on an Evolution220 UV-Visible (Thermo). NMR spectra were recorded employing Bruker 500 MHz instruments. The fluorescence imaging of cells was acquired on an Olympus fluorescent microscope (Fluo View FV1000). The absorbance of 96-well plate was collected by a TECAN infinite M200 pro microplate reader. The samples of flow cytometry analysis were used BD Biosciences FACS Aria (USA). The elemental analysis was recorded by AVario Micro cube, Germany Elmentar company. HPLC data was measured on Agilent Technologies 1260 Infinity (USA). High-resolution mass spectral (HRMS) analysis was recorded by Ultra-performance liquid chromatography-high-throughput time-of-flight mass spectrometry (Waters HCLass XEVOG2XSQTof, USA) and Thermo scientific Q EXACTIVE.

3. Cell Culture

Hep G2 cells were purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Hep G2 cells were cultured in high glucose DMEM (dulbecco’s modified eagle medium) (Hyclone, USA) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin Liquid(Solarbio, China). A549 cells were cultured in high glucose DMEM (dulbecco’s modified eagle medium) (Hyclone, USA) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin Liquid(Solarbio, China). RPMI (Roswell Park Memorial Institute) 1640 Medium(Hyclone, USA) for human neuroblastoma cells (SH-SY5Y) and human normal liver cell line (HL-7702). The cells were plated on Petri-dishes according to the instructions from the manufacturer in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C.

4. Cell cytotoxicity assay

The cellular cytotoxicity of probe 1 toward the human hepatocellular carcinoma cell line (Hep G2 cells) was evaluated using the standard MTT assay. Cells at a density of 300000 cells per mL were seeded into a 96 well microplate to a total volume of 100 μL per well in a 5% CO2 incubation 24 h. In the probe toxicity test, different concentrations of probe 1 (0, 10, 15, 20, 25, 30, 35, 40 and 50 μM) were incubated with Hep G2 cells for 12 h in medium. In the Hg$^{2+}$ toxicity test, different concentrations of Hg$^{2+}$(10, 20 and 50 μM) were added and incubated for 15min. Then added 100 μL of CCK-8 (Cell Counting Kit-8) solution into each well incubation for 4h. The absorbance of the samples was measured (450 nm) with a microplate reader. The cell viability was calculated using the following equation.

$$%Valibility = \left(\frac{A_s - A_b}{A_c - A_b}\right) \times 100\%$$

Where $A_s$ is the absorbance of different experimental wells (including the Hep G2 cells, determinand, CCK-8, DMEM medium). $A_c$ is the absorbance of the control group (including Hep G2 cells, CCK-8 and DMEM medium). $A_b$ is the absorbance of blank (CCK-8 and DMEM medium).
5. Imaging of exogenous Cys in living cells

After incubation for 24h in culture dishes, Hep G2 cells were treated with probe 1 (10 μM) and incubated for 30 min at 37 °C. Then Hep G2 cells were pretreated with Cys (100 μM) for 15 min and incubated with Probe 1 for 30 min at 37 °C. After incubation, it can be used for confocal imaging. N-ethylmaleimide (NEM, 100 μM) treated Hep G2 cells for 30 min and then cells were pretreated with Cys (100 μM), Hcy (100 μM), GSH (100 μM) for 15 min and incubated with Probe 1 for 30 min at 37 °C. (λ_{ex}=440 nm, λ_{em}=500-600 nm).

6. Imaging of endogenous Cys in living cells

After incubation for 24 h in culture dishes, the reagents (except Probe 1) and treatment methods used are as follows: Cys (100 μM) was treated with Hep G2 cells for 15 min, N-ethylmaleimide (NEM, 100 μM) treated with Hep G2 cells for 30 min, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 1 mM) treated with Hep G2 cells for 30 min, different concentration (10, 20, 50 μM) of Hg^{2+} treated with Hep G2 cells 15 min and the different concentration (100, 300, 500 μM) of H_{2}O_{2} treated with Hep G2 cells 30 min. After all pretreatments were completed, the Probe 1 was incubated with Hep G2 cells 30 min for confocal imaging. All the cells were incubated at 37 °C.

7. Apoptosis experiments and flow cytometry analysis

All the tested cells were cultured in 6-well plates (2.0 × 10^{5} cells/well), and then treated with different concentration Hg^{2+} 15 min. Before harvest, washing and suspending the cells with PBS and analyzed by flow cytometry.

Hep G2 cells were inoculated into six well plates (2.0×10^{5} cells/well) and cultured according to the description in cell culture. After Hg^{2+} treated, washing the cells with the PBS and digested them into centrifuge tubes after harvest. And then suspended the cells into 195 μL binding buffer and added 5 μL PI and 5 μL Annexin V-FITC into the above solution, respectively. The cells were kept in dark place at 25 °C, 10 min. Before analyzed by flow cytometry, suspended cells with 100 μL binding buffer.

8. Detection limit

The detection limit for Cys was calculated to be 2.89 nM based on 3σ/K (where σ is standard deviation of blank solusion for 16 samples, and σ=0.049 here, k is the slope of the Fig S7B).

9. HPLC Analysis

To confirm the reaction mechanism of probe with Cys, the product from reaction was characterized by HPLC analysis (Fig S8). The probe 10 μM and then reacted with 100 μM Cys for 30 min in EtOH. The probe, fluorophore and mixture sample were filtered through a 0.22 μm membrane for HPLC analysis. Method: C18 column (5 μm, 150 mm × 4.6 mm), mobile phase was MeOH:H_{2}O 7/3. The column temperature was 25°C, and the injection volume was 10 μL. The flow rate was 1 mL/min.

Under experimental conditions the probe 1 at 25.18 min, respectively (Fig S8a). And the Compound 2(fluorophore) eluted at 10.04 min (Fig S8b). Two peaks in Fig S8c were consistent with the peaks of probe 1 and fluorophore. (DAD detector, probe 1(Fig S8a) obtained 350 nm, Compound 2(Fig S8b) and mixture sample(Fig S8c, were obtained 380 nm).

10. Reaction mechanism analysis

The probe 1 (10 μM) reacted with 100 μM Cys for 30 min in MeOH and then measured by Ultra-performance liquid chromatography-high-throughput time-of-flight mass spectrometry.
11. Synthesis of probe

Compound 1 (160 mg, 1 mmol) and 6-hydroxy-2-naphthaldehyde (172 mg, 1 mmol) was dissolved in EtOH (10 mL), several drops of piperidine was added successively to the solution. The mixture was heated at 80 °C for 30 min with stirring. And then the mixture was cooled and was poured into water. The deep red solid, compound 2, was collected by filtration. Yield, 37%.

The compound 2 (324 mg, 1 mmol) and 2, 4-dinitrobenzenesulfonyl chloride (265 mg, 1 mmol) was dissolved in DCM (10 mL), DMAP (245 mg, 2mmol ) was added successively to the solution. Upon solvent evaporation, the crude product was purified by silica gel column chromatography yield yellow solid (probe 1). Yield, 52%.

Characterization data of compound 2: ¹H NMR (500 MHz, DMSO) δ 10.01 (s, 1H), 8.06 (s, 1H), 8.03 (dd, J = 7.9, 1.6 Hz, 1H), 7.86 – 7.78 (m, 4H), 7.78 – 7.70 (m, 2H), 7.52 – 7.43 (m, 1H), 7.25 (d, J = 16.1 Hz, 1H), 7.18 – 7.09 (m, 2H), 6.47 (s, 2H) ¹³C NMR (126 MHz, DMSO) δ 177.41, 162.53, 157.18, 155.98, 137.46, 135.87, 134.70, 134.70, 130.70, 130.00, 129.91, 128.01, 127.37, 125.71, 125.29, 124.30, 124.04, 119.80, 119.63, 118.67, 110.11, 109.54. ESI-MS (m/z): [M-H]⁻ 313.09. Molar absorption coefficients, 3.5×10⁴ cm⁻¹ M⁻¹. HRMS: 313.0923.

Characterization data of probe 1: ¹H NMR (500 MHz, DMSO) δ 9.15 (d, J = 2.3 Hz, 1H), 8.59 (dd, J = 8.7, 2.3 Hz, 1H), 8.27 (d, J = 8.6 Hz, 2H), 8.09 – 7.97 (m, 4H), 7.91 – 7.80 (m, 3H), 7.74 (d, J = 7.9 Hz, 1H), 7.54 – 7.46 (m, 1H), 7.45 – 7.34 (m, 2H), 6.52 (s, 1H). ¹³C NMR (126 MHz, DMSO) δ 177.51, 161.97, 155.98, 152.04, 147.27, 136.37, 134.89, 134.13, 132.48, 131.73, 129.33, 127.99, 125.33, 124.02, 122.41, 121.71, 121.62, 120.29, 118.72, 110.97. ESI-MS (m/z): [M+H]⁺ 545.06. Elemental analysis (%): N: 4.841; C: 60.215; H: 3.041; S: 5.280.

Scheme S1 Synthesis of probe 1. (a) Piperidine/EtOH, reflux; (b) DMAP/DCM, rt.

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Figure S1. $^1$H NMR spectra of Compound 2.

Figure S2. $^{13}$C NMR spectra of Compound 2.
Figure S3. $^1$H NMR spectra of probe 1.

Figure S4. $^{13}$C NMR spectra of probe 1.
Figure S5. MS spectra of Compound 2.

Figure S6. MS spectra of probe 1.
Figure S7 (A) Fluorescence intensity of probe 1 (100 nM) toward different concentration Cys (500 nM, 1 μM, 2 μM, 3 μM, 4 μM, 5 μM) reaction 30min at room temperature. (B) Linear relationship of the fluorescence intensity at 546 nm against the concentration of Cys from 500 nM to 5 μM. Slit, 20 nm/20 nm.

Figure S8 HPLC analysis of probe 1 wards Cys in EtOH. a-c probe 1, Compound 2(fluorophore), probe 1 with Cys.

Figure S9 HRMS spectra for the reaction of probe 1 and Cys.
Figure S10 Fluorescence intensity of probe 1 (10 μM) with Cys (100 μM) reaction in different proportion MeOH:HEPES solutions. λex = 370 nm.

Scheme S2 The proposed reaction mechanism of probe 1 and Cys.

Figure S11 Fluorescence intensity of probe 1 in different ion strength solutions (HEPES:Ethanol 3:2, reaction at room temperature). λex = 370 nm.
Figure S12 Cytotoxicity assay. Hep G2 cells were treated with probe 1 (1-9: 0, 10, 15, 20, 25, 30, 35, 40, 50μM). The experiments were repeated five times and the data were shown as mean (± s.d.).

Figure S13 Cellular co-localization imaging. Probe 1(10 μM) incubation 30 min, MitroTracker Deep Red FM(100 nM) 30 min, Hoechst 33342(one drop) 20 min, a-e, green channel(probe 1, λex=440 nm, λem=500-600 nm), red channel (MitroTracker Deep Red FM, λex=635 nm, λem=655-755 nm), blue channel(Hoechst 33342, λex=405 nm, λem= 420-480 nm), overlay of three channels, Intensity profile of interesting regions (red arrow) in overlapped images in (d). Scar bar 20μm.

Figure S14 Confocal imaging of the probe 1 in different cell lines. The cells incubated with probe 1(10 μM) for 30 min in 37 °C. Scar bar 20 μm.
**Figure S15** Nuclear imaging under Hg$^{2+}$ stimulation. The cells pre-incubated with Hg$^{2+}$ for 15 min and then incubated with probe 1 (10 μM) for 30 min, Hoechst 33342 (one drop) for 20 min. Green channel $\lambda_{ex}=440$ nm, $\lambda_{em}=500-600$ nm; Blue channel $\lambda_{ex}=405$ nm, $\lambda_{em}=420-480$ nm. Scar bar 20μm.

**Figure S16** Evaluation the cell damage caused by different concentrations of Hg$^{2+}$. Apoptosis analyzed by Annexin V/FITC, Q1-Q4: necrotic, late apoptosis, viable, early apoptosis. A-D, control, 10 μM Hg$^{2+}$, 20 μM Hg$^{2+}$, 50 μM Hg$^{2+}$. Hg$^{2+}$ incubated with Hep G2 cells for 15 min.
Figure S17 Cytotoxicity assay of different concentration Hg$^{2+}$. Hg$^{2+}$ was incubated with Hep G2 Cells for 15 min.

References:

