Supporting Information

Ratiometric Fluorescent Dye for the Detection of Glutathione in Live Cell and Liver Cancer Tissue

Duanting Zhai, Sung-Chan Lee, Seong-Wook Yun, Young-Tae Chang

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Materials and Methods

All the chemicals and solvents were purchased from commercial source, and used without further purification. Analytical characterization was performed on a HPLC-MS (Agilent-1200 series) with a DAD detector and a single quadrupole mass spectrometer (6130 series) with an ESI probe. $^1$H-NMR and $^{13}$C-NMR spectra were recorded on Bruker Avance 300 NMR and 500 NMR spectrometers, and chemical shifts are expressed in parts per million (ppm) and coupling constants are reported as a $J$ value in Hertz (Hz). High resolution mass spectrometry (HRMS) data was recorded on a Micromass VG 7035 (Mass Spectrometry Laboratory at National University of Singapore (NUS)). Spectroscopic and quantum yield data were measured on spectroscopic measurements, performed on a fluorometer and UV/VIS instrument, Synergy 4 of Bioteck Company. The slit width was 1 nm for both excitation and emission, and the data analysis was performed using GraphPrism 5.0.

1. Characterization of Glutathione Green

HPLC-MS characterization of Glutathione Green. (left) chromatograms (descending order) at 500 nm and 560 nm; (right) ESI-MS positive spectra. HPLC conditions: A: H$_2$O-HCOOH: 99.9:0.1. B: CH$_3$CN-HCOOH: 99.9:0.1; gradient 30% B to 100% B (5 min), isocratic 100% B (2.5 min). Reversephase Phenomenex C$_{18}$ Luna column (4.6 x 50 mm) 3.5 μm, flow rate: 1.2 mL/min.

HRMS m/z ($C_{22}H_{20}BCl_3F_2N_2O_3$) calculated: 514.0601 found: 495.0534 (M-F).

$^1$H NMR (300 MHz, CDCl$_3$): 7.40 (d, $J=16.2$ Hz, 1H), 7.04 (d, $J=16.2$ Hz, 1H), 7.01 (s, 1H), 6.84 (d, $J=3.9$ Hz, 1H), 6.64 (s, 1H), 6.51 (d, $J=3.3$ Hz, 1H), 6.29 (d, $J=3.9$ Hz, 1H), 6.10 (d, $J=3.3$ Hz, 1H), 4.78 (s, 2H), 3.40 (t, $J=7.5$ Hz, 2H), 2.96 (t, $J=7.5$ Hz, 2H), 2.40 (s, 3H), 2.27 (s, 3H).

$^{13}$C NMR (75.5 MHz, CDCl$_3$): 171.1, 155.9, 151.1, 144.6, 137.2, 129.5, 126.5, 125.6, 124.2, 121.2, 116.7, 116.2, 115.5, 114.9, 110.4, 109.2, 93.9, 74.1, 31.9, 23.8, 22.7, 11.3.
2. Cell Culture, Tissue Preparation and Imaging Experiments

(1) Cell Culture:

3T3 fibroblast cells were grown on cell culture Petri dishes in Dulbecco’s Modified Eagle Medium (Sigma) with 10% newborn calf serum and 5 mM L-glutamine and 5 mg/mL gentamicin. Cell cultures were maintained in an incubator at 37 °C with 5% CO₂. Cells were cultured in glass bottom, 96-well black plates for imaging experiment, 24-36 hour prior to conduction of experiments.

(2) Tissue preparation

Frozen tissue sections (10 μm) were prepared on a cryostat and mounted on lysine coated slide glasses. After drying for 30 minutes at room temperature, the tissues were incubated with 2 μM of Glutathione Green diluted in PBS for 1 hr and briefly washed with PBS. Then the tissues were mounted with PBS containing 1 μg/ml Hoechst 33342 and cover slipped.

(3) Imaging Experiments.

Glutathione Green stock solution in DMSO (5 mM) was added directly to the cell culture wells to reach the desired concentration. After 1 hour incubation at 37°C, cells were subjected for imaging with an automated fluorescence microscope ImageXpress Micro (Molecular Devices). FITC Long Pass (ex 450-490 nm, em 515 nm) and Texas Red filters were used for fluorescence image acquisition. For tissues microscope images were acquired by an inverted fluorescence microscope Ti (Nikon) using DAPI, FITC and Texas Red filters. Green and red images were merged and red/green ratio was viewed using NIS Element software.
3. Proposed mechanism of the interaction between Glutathione Green and GSH.

The LCMS data indicated the molecular weight of the orange intermediate (1) and green product (2). Based on the spectrum property and MS data, we propose the reaction between Glutathione Green and GSH occurred as shown in Scheme S1. It is known that furan ring can be opened under certain conditions.\(^\text{[1]}\) The ring-open product (1) correlates with orange fluorescence and readily undergoes Michael addition by GSH to form the green product (2) (Figure S7). However, due to the poor stability of the product in organic solvent, it could not be separated.

Scheme S1 Proposed scheme of Glutathione Green with GSH.

Reference

Figure S1 Structure of 9 GSH probes and fluorescent response of these 9 probes at 520 nm after incubation with GSH for 30 min in HEPES buffer (20 mM, pH=7.4) under excitation at 470 nm.

Figure S2 Normalized spectrum of the absorbance (red line) and fluorescence (blue line) of Glutathione Green.

Figure S3 Fluorescence response of Glutathione Green (10 μM) incubated with different thiol compounds (5 mM) after 30 min in HEPES buffer (20 mM, pH=7.4) under excitation at 470 nm.
Figure S4 Kinetic measurement by fluorescence of Glutathione Green (10 µM) incubated with GSH (5 mM). Pseudo-first order reaction constant to Glutathione Green is 2.5 s⁻¹.

Figure S5 Time dependent fluorescent response of Glutathione Green (10 µM) to GSH (2 mM) in HEPES buffer (20 mM, pH=7.4) with different percentage of DMSO (1%, 5%, 10%, 25% and 50% from top to down).
Figure S6 HPLC-MS characterization of Glutathione Green (upper). HPLC-MS characterization of Glutathione Green incubated GSH in 50% DMSO for 10 min (middle). HPLC-MS characterization of Glutathione Green incubated GSH in 50% DMSO for 2h (lower). The resulting new peaks have a mass of 840.0 [M+H] (mono-substitution) (middle), 1147.2 [M+H] (di-substitution), 574.2 [(M+2H)/2] (di-substitution; not shown here) (lower), which are corresponding to structure 1 and 2, respectively.
**Figure S7** Cytotoxic effect of Glutathione Green in 3T3, HeLa, Chang and HepG2. The cytotoxic effect of Glutathione Green was tested by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay using the CellTiter 96 nonradioactive cell proliferation colorimetric assay kit (Promega) on mESC cells. First, the cells (1 X 10^4 cells in 100 µl of media) were seeded onto 96 well plate and then different concentrations (0 µM, 2 µM, 5 µM, 10 µM and 50 µM) of Glutathione Green was added to the cells on the following day and incubated them at 37°C for 24h. After 4 h, 20 µl of MTS solution was added to each well and incubated for another 1 hours before the absorbance was measured at 490 nm. The same experiments were done for 10 h and 24 h. The control cells are 100% alive without compound condition. At least 70% of the Glutathione Green treated cells are alive after 24 h of incubation at 10 µM concentration. This indicates that the Glutathione Green is nontoxic to different cell lines.
Figure S8 GSH concentration in cell extract calculated by using commercial GSH fluorometric kit and Glutathione Green.
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**\(^1\)H NMR (300 MHz, CDCl\(_3\))**

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**COSY NMR (500 MHz, CDCl\(_3\))**
COSY NMR (500 MHz, CDCl₃)
NOSEY NMR (500 MHz, CDCl₃)
HMQC NMR (500 MHz, CDCl₃)

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HMBC NMR (500 MHz, CDCl₃)

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