Electronic Supplementary Information

for

GGCT fluorogenic probe:
Design, synthesis and application to cancer-related cells

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**Fig S1.** Release of resorufin from LISA-101 with/without GGCT. In the absence of GGCT, no detectable liberation of resorufin was occurred. Conditions were same as for Fig 2A.
Fig S2. GGCT reaction of LISA-101 was stopped by the addition of DMSO (solid line) or TFA (dashed line), when LISA-101 (1), intermediate 3 and resorufin (5) exist nearly equimolar. The reaction was monitored by RP-HPLC. By adding TFA, both enzymatic reaction and following chemical reaction were terminated. By adding DMSO, enzyme reaction was terminated, but following chemical reaction uninterruptedly proceeded to convert intermediate 3 into fluorophore 5. Additionally, remaining probe 1 was stable under such conditions.
Materials and methods

General

All reagents and solvents were obtained from the Peptide Institute, Inc. (Osaka, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Watanabe Chemical Industries, Ltd. (Hiroshima, Japan) and Sigma-Aldrich Co. LLC. (St. Louis, MO). Preparative HPLC was carried out on a Shimadzu liquid chromatograph Model LC-8A (Kyoto, Japan) with a YMC-Pack ODS-A (30 x 250 mm, particle size 5 μm, pore size 12 nm) and the following solvent systems: 0.1% TFA in H$_2$O (A) and 0.1% TFA in MeCN (B) at a flow rate of 20 mL min$^{-1}$ (room temperature) with detection at 220 nm. Analytical HPLC was performed on a Shimadzu liquid chromatograph Model LC-2010CHT/SPD-M10Avp (Kyoto, Japan) with a YMC-Pack ODS-A (4.6 x 150 mm, particle size 5 μm, pore size 12 nm) and the following solvent systems: a linear gradient of CH$_3$CN (10–90% CH$_3$CN, 25 min) in H$_2$O containing 0.1% TFA at a flow rate of 1 mL min$^{-1}$ (40 °C) with detection at 220 nm. Purities are based upon area percent of the peaks detected at 220 nm. Molecular weights were measured with an ESI MS (HP1100 LC/MSD). $^1$H NMR spectra were recorded on a JEOL-ECX400 spectrometer (Tokyo, Japan) in DMSO-d$_6$ with the solvent residual peak as an internal reference. Fluorescence spectra were measured at room temperature on a Hitachi F-2500 (Tokyo, Japan). Synergy HT (BioTek Instruments, Inc., Winooski, VT) equipped with excitation filter of 530 nm/25 nm and emission filter of 590 nm/35 nm was used for measuring fluorescence intensities.

Cells

MCF-7 (breast cancer) cells were obtained from American Type Culture Collection. NHDF (Normal Human Dermal Fibroblasts Cells) were obtained from Lonza (Basel, Switzerland). Protease inhibitor cocktails and ECL detection reagents were from Nacalai Tesque (Kyoto, Japan). Anti-GGCT monoclonal antibody (clone 6.1E) was established by Kageyama et al. (ref. 1 in the main text).

MCF-7 cells were maintained in DMEM (Wako, Kyoto, Japan) supplemented with 10% FBS. NHDF were maintained according to the manufacture’s instruction. These cells were cultured in a humidified 5% CO$_2$/95% air incubator at 37 °C.
Fluorescence spectra

LISA-101/102 or resorufin (1 μM) was dissolved in pH 8 Tris·HCl buffer containing 5 v/v % DMSO at room temperature, and the fluorescence spectrum of excitation at 530 nm was immediately recorded.

GGCT assay using HPLC

At room temperature, 40 μL of Tris·HCl buffered solution (pH 8, 100 mM) of LISA-101 (0.3 mM) and 14 μL of Tris·HCl buffered solution (pH 8, 100 mM) of GGCT (0.2 mg/mL) were mixed. The reaction mixture was left at room temperature. At each desired time point, a small portion (2 μL) of mixture was directly analyzed by RP-HPLC(UV) using molar absorption coefficient ratio of LISA-101 and resorufin (1 : 1 at 391 nm), which was determined by NMR and HPLC(UV).

GGCT assay using a fluorometer (plate reader)

In the case of measuring activity of recombinant GGCT, 100 μL of tris·HCl buffered solution (pH 8, 100 mM) containing LISA-101 (5 μM) and GGCT (5 – 200 ng) was incubated at 37 °C for 30 min, and then, DMSO (100 μL) was added to terminate the enzymatic reaction. Following additional incubation at 37 °C for 60 min to complete the chemical reaction, the fluorescence intensity was monitored with a plate reader (Synergy HT). Independently, a standard curve of resorufin was established using 50% DMSO-containing tris·HCl buffered solution (pH 8, 100 mM).

In the case of measuring GGCT activity of cell lysates, the lysate instead of GGCT solution was used. The lysates was prepared as follows: NHDF cells (5 x 10^5 cells) on the dish were washed with PBS, harvested using a cell scraper in 100 μL of tris·HCl buffered solution (pH 8, 100mM) containing 0.5% of protease inhibitor cocktail and 0.1% of Triton X-100, and crushed by sonication.

Monitoring GGCT activity of cancer-related cells

Cells were crushed by sonication, and lysate thus obtained was adjusted to contain 2 mg/mL of total
protein with the aid of standard BCA method. Then, 3 μL of HEPES buffered solution (pH 8, 100 mM) of LISA-101 (1 mg/1.8 mL) and 28 μL of cell lysate were mixed. After 1 h at room temperature, the reaction mixture was filtered and analyzed by RP-HPLC(UV) using molar absorption coefficient ratio of LISA-101 and resorufin (1 : 1 at 391 nm).

Synthesis

**LISA-101** (1) \{H-Glu\[A\textsubscript{2}bu(N\textgamma-Et-N\textgamma-CO-resorufin)-OH]-OH\}

Boc-A\textsubscript{2}bu-OrBu•HCl (970 mg, 3.12 mmol), 2,4-dinitrobenzenesulfonyl chloride (DNs-Cl, 1.25 g, 4.68 mmol) and K\textsubscript{2}CO\textsubscript{3} (2.16 g, 15.6 mmol) were dissolved in THF-H\textsubscript{2}O (2 : 1, 30 mL) and stirred for 3 h at room temperature. Then, EtOAc was added, and the organic layer was washed successively with sat. NaHCO\textsubscript{3} aq. (x 3), 0.5 N HCl (x 3) and brine (x 2). The organic layer was dried over MgSO\textsubscript{4} and the solvent was removed in vacuo. The resulting solid (1.65 g) of Boc-A\textsubscript{2}bu(N\textgamma-DNs)-OrBu was used without purification. (DNs: 2,4-dinitrobenzenesulfonyl)

Boc-A\textsubscript{2}bu(N\textgamma-DNs)-OrBu (1.57 g), ethyl iodide (374 μL, 4.68 mmol) and K\textsubscript{2}CO\textsubscript{3} (647 mg, 4.68 mmol) were dissolved in DMF (10 mL) and stirred overnight at room temperature. Then, the mixture was diluted with EtOAc, and the organic layer was washed successively with sat. NaHCO\textsubscript{3} aq. (x 3), 0.5 N HCl (x 3) and brine (x 2). The organic layer was dried over MgSO\textsubscript{4} and the solvent was removed in vacuo. The resulting solid (1.49 g) of Boc-A\textsubscript{2}bu(N\textgamma-DNs-N\textgamma-Et)-OrBu was used without purification.

Dioxane (7 mL) containing 5.7 N HCl was added into a dioxane solution (5 mL) of Boc-A\textsubscript{2}bu(N\textgamma-DNs-N\textgamma-Et)-OrBu (1.49 g), and the mixture was stirred for 1 h at 0 °C. Then, EtOAc and sat. NaHCO\textsubscript{3} aq. were added, and the organic layer was washed successively with sat. NaHCO\textsubscript{3} aq. (x 2) and brine (x 2). The organic layer was dried over MgSO\textsubscript{4} and the solvent was removed in vacuo. The resulting solid (1.00 g) of H-A\textsubscript{2}bu(N\textgamma-DNs-N\textgamma-Et)-OrBu was used without purification.

EDC•HCl (487 mg, 2.54 mmol) was added into a DMF solution (7 mL) of H-A\textsubscript{2}bu(N\textgamma-DNs-N\textgamma-Et)-OrBu (1.00 g, 2.31 mmol), Cbz-Glu-OrBu (1.26 g, 2.43 mmol) and HOBt (344 mg, 2.54 mmol), and the mixture was stirred overnight at room temperature. Then, the mixture was diluted with EtOAc, and the organic layer was washed successively with sat. NaHCO\textsubscript{3} aq. (x 2), 0.5 N HCl (x 2) and brine (x 2). The organic layer was dried over MgSO\textsubscript{4} and the solvent was removed in vacuo. The residue was purified by HPLC (H\textsubscript{2}O-MeCN system containing 0.1% TFA) to afford Cbz-Glu[A\textsubscript{2}bu(N\textgamma-DNs-N\textgamma-Et)-OrBu]-OrBu as a lyophilized amorphous powder (320 mg,
0.426 mmol). Purity: 98.6% (220 nm); MS (ESI) calcd. for (M+H)+: 752.3, found: 752.3. Cbz-Glu[A_2bu(N'-DNs-N'-Et)-OtBu]-OtBu (320 mg, 0.426 mmol) was dissolved in DMF (10 mL) containing thioglycolic acid (3 mL), and the mixture was stirred overnight at room temperature. Then, the mixture was diluted with EtOAc, and the organic layer was washed successively with sat. NaHCO_3 aq. (x 2) and brine (x 2). The organic layer was dried over MgSO_4 and the solvent was removed in vacuo. The residue was purified by HPLC (H_2O-MeCN system containing 0.1% TFA) to afford Cbz-Glu[A_2bu(N'-Et)-OtBu]-OtBu as a lyophilized oil (65.0 mg). Purity: 97.2% (220 nm); MS (ESI) calcd. for (M+H)+: 522.3, found: 522.3.

Finally, bis(trichloromethyl)carbonate (triphosgene, 12.0 mg, 41.0 μmol), resorufin (26.1 mg, 122 μmol) and N,N-diisopropylethylamine (DIEA, 22.0 μL, 126 μmol) was dissolved in CH_2Cl_2 (0.5 mL) and THF (1 mL) and stirred for 10 min under Ar atmosphere at room temperature. Then, THF (3 mL) solution of Cbz-Glu[A_2bu(N'-Et)-OtBu]-OtBu (53.0 mg, 102 μmol) and DIEA (66.0 μL, 379 μmol) was added into the solution, and the mixture was additionally stirred for 1 h under Ar atmosphere. The solution was diluted with EtOAc and washed successively with 0.5 N HCl (x 2) and brine (x 2). The organic layer was dried over MgSO_4 and the solvent was removed in vacuo. The resulting oil {Cbz-Glu[A_2bu(N'-iPr-N'-CO-resorufin)-OtBu]-OtBu} was dissolved in TFA (3.8 mL) containing triisopropylsilane (0.2 mL), and the solution was stirred overnight. Then, the solvent was removed in vacuo. The residue was dissolved in H_2O (10 mL), washed with CHCl_3 (x 3) and purified by RP-HPLC (H_2O-MeCN system containing 0.1% TFA). The desired fractions were collected and lyophilized to afford a TFA salt of the title compound as an orange amorphous powder (6.5 mg, 10 μmol). Purity: 99.2% (220 nm); ^1H NMR (DMSO-d6 containing 2% D_2O and 3% CF_3COOD, 400 MHz) 7.84 (d, J = 8.8 Hz, 1H), 7.54 (d, J = 10 Hz, 1H), 7.34 (dd, J = 15, 2.4 Hz, 1H), 7.21 (dt, J = 8.8, 8.8, 2.4 Hz, 1H), 6.82 (dd, J = 10, 2.0 Hz, 1H), 6.30 (d, J = 2.0 Hz, 1H), 4.30–4.21 (m, 1H), 3.98–3.87 (m, 1H), 3.59–3.19 (m, 4H), 2.42–2.24 (m, 2H), 2.17–1.80 (m, 4H), 1.16 (dt, J = 32, 6.8, 6.8 Hz, 3H); MS (ESI) calcd. for (M+H)+: 515.2, found: 515.2.

LISA-102 {H-Glu[A_2bu(N'-iPr-N'-CO-resorufin)-OH]-OH}

Pd/C (5% Pd) was added (120 mg) to the stirring solution of Boc-A_2bu-OtBu·HCl (353 mg, 1.14 mmol) in MeOH (20 mL) and acetone (10mL), and the reaction mixture was stirred under H_2 gas of 8.5 atm overnight at room temperature. The catalyst was filtered off and the solvent was removed in vacuo. The resulting oil (592 mg) of Boc-A_2bu(N'-iPr)-OtBu was used without purification. Then, Boc-A_2bu(N'-iPr)-OtBu was dissolved in 1,4-dioxane containing 5.7 N HCl (2 mL) and
MeOH (1 mL), and the mixture was stirred for 1h. The mixture was neutralized with N,N-diisopropylethylamine (DIEA), diluted with NMP (2 mL) and concentrated in vacuo to afford the NMP solution of H-A₂bu(N''-iPr)-OtBu.

After that, Boc-Glu(OSu)-Otbu (661 mg, 1.65 mmol) and DIEA (60 μL, 0.35 mmol) was added and the mixture was stirred overnight at room temperature. The mixture was directly purified by HPLC (H₂O-MeCN system containing 0.1% TFA) to afford Boc-Glu[A₂bu(N''-iPr)-Otbu]-Otbu as a lyophilized amorphous white powder (198 mg, 0.322 mmol). Purity: 98.4% (220 nm); ¹H NMR (DMSO-d₆, 400 MHz) δ 8.25 (d, 1H, J = 7.6 Hz), 7.13 (d, 1H, J = 7.6 Hz), 4.28–4.18 (m, 1H), 3.82–3.72 (m, 1H), 3.29–3.21 (m, 1H), 3.00–2.80 (m, 2H), 2.20 (t, 2H, J = 7.6 Hz), 2.07–1.64 (m, 4H), 1.44–1.31 (m, 27H), 1.19 (d, 6H, J = 6.4 Hz); MS (ESI): calcd. for (M + H)⁺: 502.3, found: 502.3.

Finally, bis(trichloromethyl)carbonate (triphosgene, 19.0 mg, 64.0 μmol), resorufin (41.0 mg, 194 μmol) and DIEA (35.0 μL, 196 μmol) was dissolved in CH₂Cl₂ (10 mL) and stirred for 15 min under Ar atmosphere at room temperature. Then, THF (10 mL) solution of Boc-Glu[A₂bu(N''-iPr)-Otbu]-Otbu (100 mg, 162 μmol) and DIEA (105 μL, 588 μmol) was added into the solution, and the mixture was additionally stirred for 1 h under Ar atmosphere. The solution was diluted with EtOAc and washed successively with 0.5 N HCl (x 3) and brine (x 2). The organic layer was dried over MgSO₄ and the solvent was removed in vacuo. The resulting solid {Boc-Glu[A₂bu(N''-iPr-N''-CO-resorufin)-Otbu]-Otbu} was dissolved in TFA (2 mL) containing H₂O (0.1 mL), and the solution was stirred for 2.5 h. Then, the solvent was removed in vacuo. Finally, the residue was dissolved in H₂O (10 mL), washed with CHCl₃ (x 3) and purified by RP-HPLC (H₂O-MeCN system containing 0.1% TFA). The desired fractions were collected and lyophilized to afford a TFA salt of the title compound as an orange amorphous powder (6.8 mg, 11 μmol). Purity: 99.3% (220 nm); ¹H NMR (DMSO-d₆ containing 3% D₂O and 3% CF₃COOD, 400 MHz) δ 7.83 (d, J = 8.4 Hz, 1H), 7.54 (d, J = 10 Hz, 1H), 7.37–7.28 (m, 1H), 7.21 (dd, J = 8.8, 2.4 Hz, 1H), 6.82 (dd, J = 10, 2.4 Hz, 1H), 6.28 (d, J = 2.0 Hz, 1H), 4.31–4.02 (m, 2H), 3.97–3.84 (m, 1H), 3.46–3.10 (m, 2H), 2.38–2.24 (m, 2H), 2.14–1.80 (m, 4H), 1.30–1.09 (m, 6H); MS (ESI): calcd. (M + H)⁺: 529.2, found: 529.2.
NMR

LISA-101

LISA-102

FILE 40e_JAN_27_2014_PROTON-3.a
COMMT LISA-101 DMSO 600 plus D2O

FILE 40e_JAN_24_2014_PROTON-3.a
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