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A Fluorogenic Probe for β -Galactosidase Activity Imaging in Living Cells

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Abstract

A cell permeable fluorescence turn-on probe, **AcGQCy7**, was developed to image β -galactosidase activity in living cells. Once internalized by β -galactosidase-expressing cells, the probe was hydrolyzed into a highly fluorescent molecule, and the fluorescent signal was retained in mitochondria for several days. This resulted in a long-lasting and strong β -galactosidase-dependent intracellular fluorescent signal with little background fluorescence in the culture media.

Keywords

β -galactosidase; β -galactopyranoside; fluorogenic; cyanine dye; retro-Knoevenagel reaction; thiol

INTRODUCTION

β -Galactosidase (β -Gal), an enzyme that catalyzes the hydrolysis of a terminal beta-D-galactose residue, is commonly used as a gene reporter to examine transcription and transfection efficiencies.^{1–7} Several imaging methods, such as single photon emission computed tomography (SPECT),⁸ positron emission tomography (PET),⁹ chemiluminescence,^{10, 11} and fluorescence, have been developed to assess β -gal activity *in vivo*. SPECT and PET are sensitive, capable of visualizing gene expression tomographically, suitable for deep tissue imaging and likely advanced to clinical applications. Fluorogenic probes are excellent agents to image β -Gal enzymatic activity noninvasively in living cells, as they emit fluorescence only after enzymatic activation. Examples include fluorescein di- β -galactopyranoside,¹² 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)- β -galactopyranoside (DDAOG),¹³ TokyoGreen- β -galactopyranoside (TG- β -Gal),¹⁴ and hydroxymethyl-*N,N*-diethylrhodol- β -galactopyranoside (HMDER- β -Gal).¹⁵ However, a major drawback of these probes is that hydrolyzed fluorescent products tend to rapidly diffuse across cell membranes. Therefore, the intracellular fluorescence signals are usually weak and poorly defined. Since strong background fluorescence caused by free dyes is present in extracellular media, thorough washing of cells is required before imaging. In fact, such repetitive cell washing is not only time-consuming but also harmful to cells. Here, we reported a fluorogenic probe, **GQCy7**, for detecting β -Gal activity in a cell-free solution (Fig. 1), and its derivative, **AcGQCy7**, the

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Supporting information available:

General procedures, absorption spectra and dynamic of hydrolysis of GQCy7 by β -galactosidase, mass spectra analysis of nucleophilic reaction, and comparison of cell labeling with AcGQCy7 or compound **8**. This material is available free of charge on the Internet at <http://pubs.rsc.org>.

fully acetylated form of **GQCy7**, for imaging β -Gal activity in cells. **AcGQCy7** offered a significant advantage for intracellular β -Gal imaging studies, because its hydrolyzed fluorescent product was well-retained inside living cells. The collected data demonstrated that **AcGQCy7** reported β -galactosidase activity clearly in cultured cells. Importantly, this probe has favorable characteristics for intracellular imaging, including (a) high cell compatibility, (b) efficient cell entry, (c) a strong β -Gal-associated fluorescent signal in the visible region, and (d) prolonged retention of the intracellular fluorescence signal. None of the previously reported fluorescent probes contain all of these properties^{12–16}.

EXPERIMENTAL PROCEDURES

Materials and Instruments

β -Galactosidase from *Escherichia coli* was purchased from Sigma Aldrich (St Louis, MO). C6 and C6/LacZ glioma cancer cells were purchased from ATCC (Manassas, VA). Mito Tracker[®] Green FM (M7514) and ER Tracker[™] Blue-White DPX (E12353) were purchased from Life Technologies (Carlsbad, CA). Dulbecco's Modified Eagle Medium (DMEM), Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS) buffer, fetal bovine serum (FBS), penicillin and streptomycin (PEN-STREP), L-Glutamine, and non-essential amino acids (NEAA) were purchased from GIBCO, Life Technologies (Carlsbad, CA). UV spectra were recorded with a Varian 50 Bio UV-visible spectrometer. Fluorescence emission and excitation spectra were recorded with a FluoroMax-4 Spectrofluorometer (Horiba Scientific, Edison, NJ). Cellular images were acquired with an inverted fluorescence microscope (Olympus IX81). Proton (¹H) and carbon (¹³C) nuclear magnetic resonance (NMR) spectra were obtained with a Bruker 600 MHz and 150 MHz magnetic resonance spectrometer, respectively. Solvent peaks of CDCl₃ at 7.26 ppm and 77.1 ppm were used as internal standards for ¹H and ¹³C NMR, respectively.

Synthesis of Compound 1

To a stirring solution of compound **A** (50 mg, 0.11 mmol) in anhydrous DMSO (0.22 mL) in a round bottom flask at room temperature, cyanuric chloride (11 mg, 0.55 mmol) was added. The mixture was stirred at room temperature for 40 min. The solution was diluted with EtOAc (25 mL). Then the organic solution was washed with H₂O (10 mL $\times$ 5), brine (10 mL $\times$ 1). The organic phase was dried by anhydrous Na₂SO₄, which was removed by filtration through C-sized sintered funnel. The filtrate was concentrated and the residue was purified by flash column chromatography eluted with Hexane:EtOAc (2:1) to yield the desired product as a white solid (43 mg, 82%). *R*_f = 0.45 (Hexane/EtOAc = 1:1). ¹H NMR (CDCl₃, 600 MHz) δ 7.36 (d, *J* = 8.4 Hz, 2 H), 6.98 (d, *J* = 8.4 Hz, 1 H), 5.48 (dd, *J* = 10.2, 7.8 Hz, 1 H), 5.45 (d, *J* = 3.0 Hz, 1 H), 5.10 (dd, *J* = 10.2 Hz, 3.0 Hz, 1 H), 5.04 (d, *J* = 7.8 Hz, 1 H), 4.55 (s, 2 H), 4.23–4.20 (m, 1 H), 4.17–4.14 (m, 1 H), 4.07–4.05 (m, 1 H), 2.18 (s, 3 H), 2.06 (s, 6 H), 2.01 (s, 3 H). ¹³C NMR (CDCl₃, 150 MHz) δ 170.6, 170.5, 170.3, 169.6, 157.1, 132.7, 130.3, 117.3, 99.7, 71.3, 71.0, 68.8, 67.1, 61.6, 46.0, 21.0, 20.9 (2), 20.8. LRMS calculated for (M + Na⁺) C₂₁H₂₅ClNaO₁₀⁺, 495.1, 497.1, found 495.0, 497.0.

Synthesis of Compound 2

A solution of compound **1** (55.0 mg, 0.116 mmol), 4-hydroxyisophthalaldehyde (24.0 mg, 0.162 mmol) and Ag₂O (38.0 mg, 0.162 mmol) in solvent CH₃CN (2.0 mL) was stirred at room temperature for 16 h. The mixture was filtered through celite and the pad was washed with EtOAc (100 mL). The combined organic solvent was washed with Na₂CO₃ (5%, 25 mL $\times$ 3), water (25 mL $\times$ 1) and brine (20 mL $\times$ 1), the organic phase was dried over anhydrous Na₂SO₄. The concentrated residue was purified by flash column on Si₂O eluted with Hexane:EtOAc (1:1) to yield the desired product as a white solid (68 mg, 99%). ¹H NMR (CDCl₃, 600 MHz) δ 10.48 (s, 1 H), 9.92 (s, 1 H), 8.32 (s, 1 H), 8.08 (d, *J* = 7.8 Hz, 1

H), 7.37 (d, $J = 8.4$ Hz, 2 H), 7.19 (d, $J = 8.4$ Hz, 1 H), 7.04 (d, $J = 8.4$ Hz, 1 H), 5.49-5.44 (m, 2 H), 5.22 (s, 2 H), 4.23-4.20 (m, 1 H), 4.16-4.13 (m, 1 H), 4.09-4.05 (m, 1 H), 2.16 (s, 3 H), 2.05 (s, 3 H), 2.03 (s, 3 H), 2.00 (s, 3 H); ^{13}C NMR (CDCl_3 , 150 MHz) 190.2, 188.6, 170.4, 170.3, 170.2, 169.5, 165.0, 157.2, 135.7, 132.0, 130.0, 129.3, 125.3, 117.4, 113.7, 99.5, 71.2, 70.9, 70.7, 68.7, 66.9, 61.4, 20.8, 20.7 (2), 20.6. LRMS calculated for $(\text{M} + \text{Na})^+ \text{C}_{29}\text{H}_{30}\text{NaO}_{13}^+$, 609.2, found 609.1.

Synthesis of Compound AcGQCy7

A mixture of **2** (26 mg, 0.044 mmol), 1,2,3,3-tetramethyl-3H-indolium iodide (27 mg, 0.089 mmol), NaOAc (13 mg, 0.016 mmol) in Ac_2O (0.4 mL) was stirred at 80 °C for 30 min. The solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and the insoluble NaOAc was removed by filtration through regular filter paper. The filtrate was concentrated, and then was dissolved in a small amount of CH_2Cl_2 again. The product was triturated out of the solution with addition of ether as light red-yellow solid. The crude product was further purified by flash column eluted with CH_2Cl_2 :MeOH (10:1) to yield the desired product as a red-brown solid (33 mg, 65%). ^1H NMR (CDCl_3 , 600 MHz) 9.82 (s, 1 H), 9.11 (d, $J = 8.4$ Hz, 1 H), 8.85 (d, $J = 16.2$ Hz, 1 H), 8.68 (d, $J = 16.2$ Hz, 1 H), 8.32 (d, $J = 16.2$ Hz, 1 H), 8.17 (d, $J = 16.2$ Hz, 1 H), 7.61-7.51 (m, 8 H), 7.51 (d, $J = 10.2$ Hz, 2 H), 7.40 (d, $J = 8.4$ Hz, 1 H), 7.10 (d, $J = 10.2$ Hz, 2 H), 5.51 (dd, $J = 10.2$ Hz, 2 H), 5.22 (s, 2 H), 4.23-4.20 (m, 1 H), 4.16-4.13 (m, 1 H), 4.09-4.05 (m, 1 H), 2.16 (s, 3 H), 2.05 (s, 3 H), 2.03 (s, 3 H), 2.00 (s, 3 H). ^{13}C NMR (CDCl_3 , 150 MHz) 183.6, 183.1, 170.5, 170.3, 170.2, 169.7, 162.8, 157.4, 154.5, 148.8, 144.0, 143.4, 141.5, 141.4, 140.1, 135.8, 130.3, 130.2, 129.9 (2), 129.8, 129.5, 128.7, 123.6, 123.0, 122.9, 117.5, 114.6, 114.4, 114.2, 114.1, 112.9, 99.6, 71.4, 71.3, 70.8, 68.8, 67.0, 61.4, 53.0, 52.5, 37.6, 37.5, 27.3, 27.1, 21.0, 20.9, 20.8, 20.7. LRMS calculated for $(\text{M}^{2+} + \text{MeO})^+ \text{C}_{54}\text{H}_{61}\text{N}_2\text{O}_{12}^+$, 929.4, found 929.3.

Synthesis of Compound GQCy7

Compound AcGQCy7 (20 mg, 0.017 mmol) was dissolved in anhydrous MeOH (0.17 mL). Then a solution of NaOMe (0.17 mL, 0.5 M in Methanol) was added dropwise to the solution. After 30 min, the mixture was directly loaded on the prep-TLC and eluted with CH_2Cl_2 :MeOH (10:1) to yield the desired product as a brown-yellow color solid (11 mg, 65%). LRMS calculated for $(\text{M}^{2+} + \text{MeO})^+ \text{C}_{46}\text{H}_{53}\text{N}_2\text{O}_8^+$, 761.4, found 761.2.

Staining cells with AcGQCy7

C6 and C6/LacZ glioma cancer cells were cultured in DMEM supplemented with 10% FBS, 1% PEN-STREP, 200 nM L-Glutamine, and 100 nM NEAA at 37°C in a humidified 5% CO_2 incubator. Cells (5×10^3 cells) were seeded in a 96-well plate (black wall and clear bottom, Corning Costar®, Corning Life Sciences, Pittston, PA) 1 day before treatment. Cells were washed with HBSS and incubated with **AcGQCy7** (1 μM) in HBSS for 40 min at 37°C with humidified 5% CO_2 . After incubation, cells were washed twice with HBSS and placed in fresh phenol red-free DMEM for fluorescence imaging.

Colocalization of AcGQCy7 staining with Mito or ER trackers in C6/LacZ cells

C6/LacZ cells (5×10^3 /well) were seeded in a 96-well plate in DMEM supplemented with 10% FBS, 1% PEN-STREP, 200 nM L-Glutamine, and 100 nM NEAA at 37°C for 1 day. Cells were incubated with **AcGQCy7** (1 μM) in HBSS for 40 min at 37°C in a humidified 5% CO_2 incubator. After incubation, cells were washed twice with HBSS and incubated with either Mito-tracker Green (200 nM) or ER-tracker Blue (200 nM) in HBSS for 15 min. Finally, cells were washed and placed in fresh phenol red-free DMEM for fluorescence imaging.

Fluorescence Microscopy

Cells were viewed with an inverted Olympus 8IX fluorescence microscope. Images of **AcGQCy7** were collected through the TRITC (excitation: 510–550 nm, emission: 573–648 nm) band pass filter. Images of Mito Tracker Green (MTG) were obtained through the FITC (excitation: 450–490 nm, emission: 500–550 nm) filter. Images of ER Tracker Blue were visualized with the DAPI (excitation: 325–375 nm, emission: 435–485 nm) filter.

RESULTS AND DISCUSSION

Design and synthesis

Rational design of the β -Gal-responsive fluorogenic probe, **GQCy7**, was based on a near infrared (NIR) fluorophore, **QCy7** (Figure 1), which was described in the literature.^{17, 18} **QCy7** has a π -conjugated system similar to other cyanine dyes, in which one positive charge delocalizes between two nitrogen atoms through a heptamethine chain and emits at 715 nm in PBS 7.4 buffer. It was found that the fluorescence emission property of **QCy7** could be masked by a β -galactopyranosidylated phenolic oxygen group (Figure 1). The prepared **GQCy7** probe was non-fluorescent in the NIR region, because its π -conjugated system was disrupted. Enzymatic cleavage of β -galactose by β -Gal under physiological conditions could produce an unstable phenol intermediate. Subsequent self-immolative elimination^{16, 19, 20} of 4-methylenecyclohexa-2,5-dienone resulted in the highly fluorescent product, **QCy7**. This self-immolative spacer was critical for stabilizing the whole molecule, because a direct connection between the galactose residue and **QCy7** was found to be unstable.

Probe **GQCy7** was synthesized by a four-step procedure with a known compound, **A**,²¹ as the starting material (Scheme 1). Alcohol **A** was converted into chloride **1** with 82% yield under neutral conditions with cyanuric chloride in DMSO in accordance with a previously developed protocol for rapid chlorination of benzyl alcohol.²² Synthesis of compound **2** was initially attempted by coupling compound **1** with *p*-hydroxylisophthalaldehyde in the presence of Cs₂CO₃ and catalyst Bu₄NI in DMF.²³ However, the yield was poor. An increased yield was later achieved by applying Ag₂O to the coupling reaction. The aldehyde groups on **2** were condensed with indolium to generate the acetylated probe, **AcGQCy7**, with 65% yield. Deacetylation of **AcGQCy7** was performed with NaOMe in methanol for 30 min, which resulted in the desired product, **GQCy7**, at 65% yield. **GQCy7** was purified by preparative thin layer chromatography. In summary, compounds **AcGQCy7** and **GQCy7** were readily prepared in three or four steps, respectively, with 53% and 35% total yields. These compounds were characterized by ¹H, ¹³C NMR and mass spectroscopy (see supporting information).

Detection of β -Gal enzymatic activity in buffer

The enzyme reactivity of **GQCy7** was validated with pure β -Gal in pH 7.4 buffer. The probe (300 μ M) was dissolved in HBSS in the presence or absence of β -Gal (15 U/mL) at 37°C for 30 min. The solution of **GQCy7** without β -Gal was yellow (Figure 2a) and had a single absorbance band with a maximum value at 400 nm (Figure S1a). If **GQCy7** was in contact with β -Gal, the color of the solution rapidly turned into dark blue (Figure 2a), and a broad absorbance band covering 400–580 nm was detected (Figure S1a). Under 575–605 nm excitation light, the fluorescence image showed that the solution without β -Gal had no fluorescence emission. In contrast, the solution containing β -Gal exhibited a well-observed fluorescence emission (Figure 2b). The fluorogenic conversion was completed in 30 min (Figure S1b). The fluorescence signal of probe **GQCy7** was measured in HBSS media with a spectrofluorometer. The intensity of the β -Gal-treated solution was 110-fold higher than

that of the solution without β -Gal (Figure 2c). These data indicated that probe **GQCy7** was sensitive to β -Gal in biochemical assays in buffer.

Detection of intracellular β -Gal enzymatic activity

The cellular imaging potential of β -Gal activity was examined in two cell lines, rat glial tumor C6 (control, no β -galactosidase expression) and its derivative, C6/LacZ, which constitutively expresses β -Gal. After incubating cells with **GQCy7** (1 μ M), surprisingly, no obvious fluorescence in either group of cells was observed (data not shown). It was suspected that **GQCy7**, which contained a hydrophilic galactose residue, might be too polar to pass through the cell membrane. To detect intracellular β -Gal activity, a probe must be cell-permeable. Therefore, **AcGQCy7**, which contained the fully acetylated β -galactose, was tested. Although the acetylated β -galactose could not be recognized by β -Gal, upon cell entry, the acetyl groups were expected to be rapidly removed by intracellular esterases, resulting in unblocked **GQCy7**. C6/LacZ or C6 cells were incubated with **AcGQCy7** (1 μ M) in HBSS buffer at 37°C for 40 min. As expected, a bright fluorescence signal was found only in the β -Gal-positive C6/LacZ cells and not in the β -Gal-negative C6 cells (Figure 3). Cell imaging results confirmed the hypothesis that fully acetylated **AcGQCy7** was efficiently internalized by cells followed by rapid hydrolysis of the acetyl groups as we previously demonstrated²⁴ and β -Gal-mediated hydrolysis of galactose, in which resulted in a bright intracellular fluorescence signal. Surprisingly, the intracellular fluorescent signal was not in the 570 nm excitation, and 650–700 nm emission window in contrast to the cell-free spectroscopic measurement. It was detected indeed by the TRITC filter set, whose excitation and emission is 510–560 nm, and 572–648 nm, respectively. The unexpected difference between biochemical and cellular experiments suggested that the long π -conjugated system in **QCy7** might be shortened in cells.

Mechanism of intracellular fragmentation

Examining the chemical structure of **AcGQCy7**, it was speculated that a nucleophile, such as a thiol-containing molecule,²⁵ could attack the conjugated double bond. Similar to the Michael addition, a thiol group could react with the β , γ -unsaturated iminium ion, **GQCy7**, forming intermediate enamine **3** and then rearrange to form iminium ion **4** by picking up a proton (Scheme 2). The subsequent retro-Knoevenagel condensation reaction^{26, 27} of **4** could produce thionium ion **5**, which could be further hydrolyzed into aldehyde **6** in an aqueous environment. Removal of the sugar residue in molecule **6** by β -Gal could yield fluorophore **7**. Because the π -conjugated system in structure **7** is significantly shorter than that of **QCy7**, its emission in the NIR region was lost. Comparison of the optical properties of a literature compound, fluorophore **8** ($\text{Ex}_{\text{max}} = 542$ nm, $\text{Em}_{\text{max}} = 562$ nm, MeOH),²⁸ which has a core structure similar to **7**, suggests that the intracellular compound **7** should emit light with a peak around 560 nm.

To prove this hypothesis, **GQCy7** (100 μ M) was incubated with an excess amount of cysteine (1.0 mM, 10 eq) in PBS 7.4 buffer at 37°C for 30 min (Figure 4). The reaction solution was directly examined by mass spectroscopy. The molecular mass peak at 761.3 Daltons corresponding to **GQCy7** disappeared, but a new 574.1 Dalton peak corresponding to compound **6** (Figure S2a, and S2b) was observed. This result indicated that the retro-Knoevenagel reaction proposed in scheme 2 was a fast process. Further addition of β -Gal to the reaction solution resulted in the conversion of β -galactopyranoside **6** into compound **7**. The fluorescence spectrum of the reaction mixture, which was diluted to one-tenth the initial concentration in PBS 7.4 buffer, is shown in Figure 4b. The fluorescence intensity of the solution in the presence of cysteine (100 μ M) was approximately 20-fold stronger compared to that of the control. Moreover, the fluorescence spectrum of the reaction mixture was very similar to that of compound **8** with maximum fluorescence observed at 557 nm.²⁸ This

further supported the hypothesis proposed in scheme 2. To demonstrate that biological thiols play a critical role in the retro-Knoevenagel reaction compared to primary amines on proteins, a glycine-assisted hydrolysis reaction of **GQCy7** in a thiol-free environment was tested (Figure S3a). The hydrolysis reaction with glycine was found incomplete even the reaction time was extended, as shown by the presence of the mass peak of the starting material, **GQCy7**, at 761.3 (Figure S3b). Addition of fresh cysteine (10 eq) to the glycine reaction solution allowed completion of the reaction in 30 min, as indicated by the disappearance of the starting material, **GQCy7**, from the mass spectrum (Figure S3c). These results confirmed that biological thiols are more efficient at breaking down **GQCy7**.

Long intracellular retention of fluorescence signal

To study retention of the probe, C6/LacZ cell lines were incubated with **AcGQCy7** (1 μ M) in HBSS buffer at 37°C for 40 min, and the probe solution was replaced with phenol red-free DMEM. Cells were maintained at 37°C and imaged by fluorescence microscopy at different time points without washing. Strong fluorescence was restricted within C6/LacZ cells on days 1 and 3. Little background fluorescence was detected in cell culture media (Figure 5). Importantly, cell morphology showed no signs of toxicity at any of the time points. The persistent and intense intracellular signal indicated that **AcGQCy7** is a useful probe for imaging β -Gal activity in cells over a long period of time. A well-retained probe is preferred for long-term cellular imaging studies. If fluorescent molecules were easily expelled out of cells, the intracellular fluorescence signal would be poorly defined.^{29, 30} The functional aldehyde group in the hydrolyzed product, **7**, might have promoted retention by reacting with biological primary amines. This conjugation could cause increased intracellular retention of the probe, resulting in reduced background fluorescence in culture media. However, the aldehyde and amine did not form a permanent linkage. Thus, the signal was decreased upon fixing cells in organic solvents.

Localization of fluorescent label in C6/LacZ cells

The intracellular distribution of fluorescence was not homogeneous but was confined to a specific organelle (Figure 6). No signal was observed within the nucleus. Subcellular localization of the fluorescence signal was further examined by co-staining with the mitochondria-selective dye, MTG³¹ and endoplasmic reticulum (ER) tracker blue.³² Co-localization studies showed that the red fluorescent signal from **AcGQCy7** overlapped with the MTG signal (Figure 6a–d), suggesting that the cleaved product specifically targeted mitochondria. The fluorescence signal of compound **7** did not correspond to the signal of the ER tracker (Figure 6e–g). The confined mitochondria signal is probably due to the positive charge of the hydrolyzed fluorescent product **7**. Mitochondria have negative membrane potentials and, thus, retain positively charged compounds.³³ This finding is consistent with our recent report of **QCy7** as an excellent mitochondria label.³⁴

Based on the results and the emission wavelength, non-fluorescent **AcGQCy7** appears to be converted into fluorescent **7**, which is retained within mitochondria, through a series of reactions, including the enzymatic cleavage of galactose by β -Gal. Due to the structure similarities between compound **7** and compound **8**, compound **8** might as well be a good mitochondria labeling agent. Therefore, compound **8** was acetylated and cultured with C6/LacZ cells to test this hypothesis. Intracellular fluorescence was observed within 30 min, but high background fluorescence was also detected in media (Figure S4). The signal contrast was significantly different from that obtained with **AcGQCy7** (compare to Figure 3). After washing away the background fluorescence, a weak fluorescent signal from compound **8** was found in mitochondria. These results suggest that the positively charged compound **8** could label mitochondria but with low efficiency, which may be due to poor uptake of the probe.

CONCLUSION

In summary, **GQCy7**, a fluorogenic probe that detects β -Gal activity in biochemical conditions, was designed, synthesized, and evaluated. In a cell-free assay, **GQCy7** produced a 110-fold increase in fluorescence with a peak at 680 nm in the presence of β -Gal. But it could not be used in cell assay, probably due to poor membrane permeability. In contrast, the fully acetylated form of **GQCy7**, **AcGQCy7**, was cell-permeable and suitable for imaging β -Gal activity in living cells. The hydrolyzed product of **AcGQCy7** was retained in cells, and little free probe was expelled out of the cells. These unique characteristics of **AcGQCy7** offer a wash-free advantage for imaging cellular β -Gal activity. The hypsochromic shift of the intracellular fluorescence was found thiol-dependent, in contrast to cell-free fluorescence spectra. In the presence of biological molecules that contain thiols, such as cysteine, and β -Gal, the core structure of **GQCy7** was hydrolyzed into compound **7**, which emitted fluorescence around 560 nm with excitation around 530 nm. Furthermore, the hydrolyzed product resulted in increased cellular accumulation and retention of the probe, offering a clear visualization of β -Gal activity in living cells for longer than 3 days. However, the fluorescent signal was confined to mitochondria. Although in vivo imaging of β -Gal activity has been the focus of many biological studies,^{4, 35, 36} the disappearance of NIR fluorescence in cells diminishes the potential of using **AcGQCy7** in animal studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Rational design of fluorogenic probe **GQCy7** for β -Gal sensing.

Figure 2.

(a) Light image and (b) fluorescence image (excitation: 575–605 nm, emission: >680 nm) of probe **GQCy7** (300 μ M) in HBSS solution for 30 min in the presence or absence of β -Gal. (c) Fluorescence spectra of probe **GQCy7** (10 μ M) in the presence (red) and absence (blue) of β -Gal (λ_{ex} = 570 nm) at 37°C for 30 min.

Figure 3. Imaging β -Gal activity in cells. Images of probe **AcGQCy7** (1 μ M) in C6 cells (a, b) and C6/LacZ cells (c, d) for 40 min at 37°C. Note: The fluorescent images were acquired using a TRITC filter (excitation: 510–550 nm, emission: 573–648 nm). Exposure time: 800 ms.

a **b** **Figure 4.**

Studies of thiol-induced fragmentation of **GQCy7**. (a) The proposed retro-Knoevenagel reaction of **GQCy7** with cysteine. (b) Spectra of **GQCy7** alone and **GQCy7** with cysteine and β -Gal in PBS 7.4 buffer. **GQCy7** (100 μ M) was treated with cysteine (1 mM) for 30 min followed by incubation with β -Gal for an additional 30 min. The solution was diluted 10 times prior to measurement (λ_{ex} = 490 nm).

Figure 5. Cell retention studies of AcGQCy7 probe in C6/LacZ cells. Cells were incubated with AcGQCy7 (1 μM) in HBSS for 40 min. Media were replaced with fresh DMEM and incubated for 3 days. Fluorescence images were taken on day 1 and day 3. (a, c) bright-field images and (b, d) fluorescence images.

Figure 6.

Co-registration of fluorescence signals produced by probes **AcGQCy7** (1 μ M) and Mito Tracker Green (200 nM) or ER tracker Blue (200 nM) in C6/LacZ cells. (a, e) white light images; (b) fluorescence image with Mito Tracker Green; (f) fluorescence image with ER Tracker blue; (c, g) fluorescence images with **AcGQCy7**; (d, h) merged fluorescence images.

Compounds 8 and 8-Ac

Figure 7.

Scheme 1.
Synthesis of probes **AcGQCy7** and **GQCy7**.

Scheme 2.

The proposed mechanism of transformation for probe **AcGQC7** in living cells.