Design and Development of an HBT-Based Ratiometric Fluorescent Probe to Monitor Stress-Induced Premature Senescence

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ABSTRACT: Stress-induced premature senescence (SIPS) can be induced in tumor cells by reactive oxygen species (ROS) or oncogenes. The antineoplastic drugs cause apoptosis and senescence by damaging the DNA. Although the detection of cellular senescence is important to monitor drug response during anticancer therapy, only a few probes have been studied for imaging SIPS. In this study, we developed 2-(2′-hydroxyphenyl)benzothiazole (HBT)-based fluorescent probes to determine SIPS by monitoring the oxidative stress and β-galactosidase activity. HBT is a commonly used fluorophore because of its luminescence mechanism via excited-state intramolecular proton transfer, and it has attractive properties, such as a four-level photochemical process and large Stokes shift (151 nm). A novel fluorescent probe, (2-(benzo[d][1,2-b]thiazol-2-yl)phenyl)boronic acid, was prepared for the detection of ROS, including H2O2, via the oxidation reaction of arylboronic acids to form the fluorescent phenol, HBT. In addition, to determine the enzymatic activity of β-galactosidase, a 2-(4′-chloro-2′-hydroxyphenyl)-benzothiazole (CBT)-based enzymatic turn-on probe (CBT-β-Gal) was designed and synthesized. β-Galactosidase catalyzed the hydrolysis of β-galactopyranoside from CBT-β-Gal to release the fluorescent CBT. These probes were capable of ratiometric imaging the accumulation of H2O2 and the degree of β-galactosidase activity in contrast to H2O2-untreated and H2O2-treated HeLa cells. Furthermore, these probes were successfully employed for imaging the increased levels of ROS and β-galactosidase activity in the doxorubicin-treated HeLa cells.

INTRODUCTION

Cellular senescence can be induced by telomere shortening, which causes irreversible cell cycle arrest. This mechanism plays an important role in aging, tumor suppression, and developing tumors. Numerous reports have documented that oxidative stress and oncogenes can induce stress-induced premature senescence (SIPS) in tumor cells, which differs from replicative senescence, known as passaging. Reactive oxygen species (ROS), such as the superoxide anion (O2⋅−), hydroxyl radical (OH•), and hydrogen peroxide (H2O2), despite being intracellular signals and growth stimulants, are well-known triggers of senescence. In particular, H2O2 is widely used as an exogenous inducer of cellular senescence. Excessive oxidative stress induced by H2O2 produces oxidative damage to DNA, which promotes SIPS in tumor cells. This generally results in a distinct morphology change, gene expression pattern, and secretory phenotype. The most common feature is the increased activity of the senescence-associated β-galactosidase in the G1 phase of the cell cycle. The antineoplastic drugs, such as cisplatin and doxorubicin (Dox), have been used to treat several human cancers because they trigger ROS-induced apoptosis, senescence, and DNA damage. However, escape from senescence would result in cancer cell repopulation and treatment resistance. Therefore, the identification of the senescence state is necessary to improve the response to anticancer therapy. Accordingly, the development of ratiometric fluorescent probes to monitor the concentration of ROS and β-galactosidase activity in living cells is needed to detect SIPS.

Recently, many β-galactosidase-targeting fluorescent probes have been developed to detect senescent cells. The traditional analyte, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), is widely used for chromogenic staining of β-galactosidase activity in fixed cells or tissues. Urano developed a series of rhodamine-derived probes for the detection of β-galactosidase in cultured cells and Drosophila melanogaster tissue with LacZ(+) that encodes Escherichia coli β-galactosidase activity. Recently, Cui and co-workers achieved in vivo imaging of DNA damage-induced senescence using a near-infrared fluorescent probe. However, the usage...
of fluorescent probes has not been appropriately studied for the ratiometric detection of the senescence-associated β-galactosidase activity induced by the ROS in living cancer cells. Typically, the ROS level is elevated in the case of almost all cancers and is linked to tumor development and progression.16,17 These cancer cells display increased levels of β-galactosidase, suggesting that monitoring the degree of altered levels of β-galactosidase activity and senescence-associated cellular changes that are induced by additional ROS is required.

In this study, we designed and synthesized simple and robust fluorescent probes to monitor oxidative stress and β-galactosidase activity for the detection of SIPS (Figure 1A). 2-(2'-Hydroxyphenyl)benzothiazole (HBT), despite being a small organic molecule, is a commonly used fluorophore with attractive properties, including a four-level photochemical process and a large Stokes shift as well as solubility in aqueous media, compared to those of conventional fluorophores such as fluorescein and boron dipyrromethene. This luminescence mechanism is based on the excited-state intramolecular proton transfer (ESIPT). Typically, the ESIPT fluorophores exist in an enol form in the ground state (N). Upon photoexcitation, they exhibit a dual-emission process with the tautomeric form (T*) from the normal form (N*). The excited enol form rapidly undergoes ESIPT conversion to its excited keto form. After decaying in keto emission, the ground state keto tautomer (T) undergoes retro proton transfer to ensure the regeneration of the initial enol form. Because of this photochemical process, which reduces the inner filter effect, the ESIPT-based fluorescent probes can be used as imaging agents.18 Although some of the ESIPT-based fluorescent probes have allowed the determination of the ROS and β-galactosidase activity, a suitable fluorescent probe is still needed for imaging the SIPS in tumor cells.19–24 We prepared some phenylboronic acid derivatives, as shown in Figure 1B. Among them, (2-(benzo[d][thiazol-2-yl]phenyl)boronic acid (BTPB) 1a exhibited sensitivity toward ROS subsets, such as hydroxyl radical (OH*), superoxide radical (O2•*), and H2O2 and it was successfully applied for imaging of cellular ROS in living cells with high contrast. A 2-(4′-chloro-2′-hydroxyphenyl)-benzothiazole (CBT)-based enzymatically turn-on probe (CBT-β-Gal) 4 was designed, synthesized, and used to detect the increased level of the β-galactosidase activity in the HeLa cells. CBT-β-Gal exhibited enhanced fluorescence intensity in H2O2-treated cells when compared with H2O2-untreated cells, and the fluorescence increased with increasing H2O2 concentration. In addition, these probes could be used to stain the ROS and β-galactosidase activity in the doxorubicin-treated HeLa cells.

**RESULTS AND DISCUSSION**

Because of the photostability in aqueous solution and lack of autofluorescence background, the HBT fluorophore is more advantageous for bioimaging when compared with the conventional fluorescent agents. Otsubo et al. synthesized ESIPT-based HBT derivatives exhibiting a large Stokes shift (approximately 150 nm).25 They determined that the HBT substituent pattern shifted the fluorescence wavelength. Inspired by their work, we designed HBT-based probes, BTPB derivatives, for H2O2 detection via the oxidation reaction of the arylboronic acids to transform into the fluorescent phenol. Arylboronic acids 1a–f with substitutes, such as chlorine, fluorine, and methoxy groups at the phenyl ring, were synthesized according to the reported procedures (Scheme 1). To confirm their capability to detect H2O2, we measured the time-dependent fluorescence intensities of 1a–f (100 μM) after the addition of H2O2 (1 mM) for 2 h. The fluorescence intensity was recorded at 510 nm with 356 nm excitation using a multiplate reader (Figure S1).

The fluorescence intensities of 1a without a substitute and 1b with a chlorine atom increased by 30 times in 2 h upon the addition of H2O2. 1c with a fluorine atom did not present adequate enhanced fluorescence. When 1d and 1e with a methoxy group were evaluated upon the addition of H2O2, the electron-donating effect caused a longer shift in fluorescence wavelength when compared with that observed in 1a. Unfortunately, these probes were not suitable for imaging because of their poor solubility in aqueous media. However, 1f

![Figure 1](https://dx.doi.org/10.1021/acsomega.9b04208)

**Figure 1.** (A) Mechanism of the ESIPT process. (B) Chemical structures of 1a and CBT-β-Gal 4 and the principle of ESIPT-based fluorescence for the detection of H2O2 or doxorubicin-induced cellular senescence.
exhibited strong fluorescence with absorption at 365 nm. These results implied that probe 1a could rapidly detect H₂O₂ and provide bright fluorescence for imaging. Subsequently, we examined the cytotoxicity of probe 1a using a standard cell viability assay, i.e., the WST-1 assay. As presented in Figure 2, the HeLa cells treated with concentrations of up to 50 μM of probe 1a exhibited similar viability as that exhibited by the untreated cells.

Figure 2. Cytotoxicity of probe 1a. The HeLa cells were treated with probe 1a for 24 h, and the cell viability was determined using the WST-1 assay. Data shown is an average ± SD.

The photophysical properties of probe 1a were assessed in the presence of H₂O₂ in phosphate-buffered saline (PBS) buffer (Figure 2a). The solution of probe 1a emitted a weak fluorescence (λ_em = 458 nm, Φ = 0.02) in the presence of H₂O₂. The reaction of 1a with H₂O₂ undergoes conversion to the fluorescent phenol, HBT, via ipso-oxidation. The fluorescence emission was gradually red-shifted (λ_em = 510 nm) and steadily increased upon exposure to H₂O₂ (for a period of 2 h). After 2 h, the absolute quantum yield increased to 0.21 (Figure S4A). The UV−vis absorption and fluorescence spectra of the fluorophore HBT were measured using a PBS buffer (pH 7.0). The major absorption band was at 359 nm (ε = 3178 M⁻¹ cm⁻¹), and the fluorescence maximum wavelength emitted at 510 nm (Φ = 0.32) had a large Stokes shift of 151 nm. Next, we evaluated the fluorescent wavelengths of the PBS solutions having different pH values (6.0, 6.4, 7.0, 7.4, and 8.0) (Figure S3 and Table S1). HBT was protonated to the keto form in a slightly acidic solution (pH 6.0 and 6.4), increasing the fluorescence emission at approximately 510 nm via the ESIPT process. At pH 7.0, blue and green signals could be observed. Blue fluorescence was mainly observed through enol emission in the case of physiological pH values (pH 7.4) and weak basic conditions (pH 8.0). Similarly, we analyzed the UV−vis absorption and fluorescence spectra of the fluorophore 2-(4'-chloro-2'-hydroxyphenyl)benzothiazole (CBT) relative to 1b. CBT exhibited useful photophysical properties (λ_em = 508 nm, Φ = 0.36, and ε = 3635 M⁻¹ cm⁻¹) in the case of biological imaging.

To examine the selectivity of 1a for biologically relevant ROS, the fluorescence change was measured in the presence of H₂O₂, hypochlorite (ClO⁻), tert-butylhydroperoxide (tBHP), nitrite (NO₂⁻), tert-butyl radical (•OTBu), hydroxyl radicals (•OH), and superoxide (O₂⁻) (Figure 3B). Among the ROS species, probe 1a exhibited selectivity for ROS subsets, such as H₂O₂, hydroxyl radicals (•OH), and superoxide (O₂⁻), according to the significantly enhanced fluorescence intensity. These results suggested that 1a acts as a ratiometric fluorescent probe not only for H₂O₂ but also for •OH and O₂⁻.

We examined the capability of probe 1a to detect H₂O₂ in the HeLa cells as a model of cell lines (Figure 4). After incubation with 1a (50 μM) for 30 min, the cells were treated
with H$_2$O$_2$ (200 or 400 μM) for 30 min. The imaging at an excitation with 365 nm showed enhanced fluorescence intensity in the blue channel. Imaging with 1a in the blue channel was sharper than that in the green channel. We observed a significantly enhanced fluorescence intensity for the cells cultured in the presence of H$_2$O$_2$. The fluorescence intensity was brighter for 400 μM H$_2$O$_2$ treatment than for 200 μM (Figure 4A). The fluorescence intensity was enhanced by up to approximately 7 times in cells treated with 400 μM H$_2$O$_2$ when compared with that in untreated cells. Fluorescence imaging was unsuccessful because of the poor solubility of probe 1b. We further performed the detection of endogenous ROS in HeLa cells that were incubated with probe 1a for 24 h (details are shown in Figure S7). We observed increased blue fluorescence to detect cellular ROS in living cells. Thus, probe 1a could image the cellular accumulation of ROS.

Otsubo et al. developed 2-(benzothiazol-2-yl)-phenyl-β-D-galactopyranoside (BT-β-Gal) derivatives, which can be used to assay the β-galactosidase activity.$^{25}$ We evaluated the potential of BT-β-Gal without a substitute to monitor the H$_2$O$_2$-induced β-galactosidase activity in the HeLa cells. However, an effective fluorescence signal was not recorded. We next designed the 2-(4′-chloro-2′-hydroxyphenyl)-benzothiazole (CBT)-based enzymatic turn-on probe, (CBT-β-Gal) 4. The synthetic route toward CBT-β-Gal 4 is illustrated (Scheme 2). CBT 2 was synthesized by condensation of 4-chlorosalicylaldehyde with 2-aminobenzimide according to the reported procedures. The sequential reaction of CBT with 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide produced the corresponding product 3 in a 70% yield. The hydrolysis of acetyl esters produced CBT-β-Gal.

The cytotoxicity of CBT-β-Gal 4 was evaluated using the WST-1 assay (Figure 5). The results revealed that CBT-β-Gal 4 concentrations of 25 μM had a cytotoxic effect on HeLa cells, suggesting that CBT-β-Gal 4 should be used for bioimaging at concentrations lower than 25 μM.

To confirm the capability of CBT-β-Gal 4 to detect β-galactosidase activity, we measured the time-dependent fluorescence intensity after the addition of β-galactosidase (20 U mL$^{-1}$) for 20 min with 356 nm excitation (Figure 6). As a result, CBT-β-Gal 4 emitted extremely weak fluorescence (Φ = 0.045), then the fluorescence intensity of 4 upon the addition of β-galactosidase significantly increased within 15 min. β-Galactosidase catalyzed the hydrolysis of β-galactopyranoside from CBT-β-Gal 4 and released fluorescent CBT (λ$_{em}$ = 508 nm, Φ = 0.339) (Figure S4B). Hence, probe 4 achieved rapid detection for β-galactosidase activity.

Upon exposure of cancer cells to H$_2$O$_2$, the oxidative stress induces cellular senescence and elevated β-galactosidase activity is used as a biomarker of this phenotype. However,
CBT-associated galactosidase detection kit for staining the senescence-galactosidase-positive. Initially, we performed β-culturing for 24 h, they undergo senescence and become fl with H2O2 (400 μM). These probes exhibited a large Stokes shift and pH sensitivity and could image the accumulation of ROS, which can induce cellular senescence. In addition, CBT-β-Gal 4 could be used to monitor and image ROS and β-galactosidase in response to the Dox-induced DNA damage.

**CONCLUSIONS**

We developed two ESIPT-based fluorescent probes, 1a and CBT-β-Gal 4, to detect H2O2 and β-galactosidase activity. These probes exhibited a large Stokes shift and pH sensitivity and could be used in several biological applications. Probe 1a could image the accumulation of ROS, which can induce cellular senescence. In addition, CBT-β-Gal 4 exhibited a good staining capability for β-galactosidase activity. We detected the increased β-galactosidase activity upon exposure of the HeLa cells to H2O2. Furthermore, we demonstrated the detection of ROS and β-galactosidase activity in the Dox-treated HeLa cells. Our study will be helpful in advancing the understanding of the process of aging, cellular senescence, tumor suppression, tumor development, and monitoring drug response in cancer therapies.

**EXPERIMENTAL SECTION**

**Instruments and Materials.** All NMR spectra were recorded on Varian 500PS spectrometers. 1H and 13C NMR spectra are reported as chemical shifts (δ) in parts per million (ppm) relative to the solvent peak using tetramethylsilane (1H and 13C) as an internal standard. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) were measured in hertz (Hz). NMR spectra were processed in ACD/SpectManager. High-resolution mass spectra (HRMS, m/z) were obtained on a JEOL JMS-700N for FAB using m-nitrobenzyl alcohol as the matrix or on a JEOL JMS-T100TD for electrospray ionization (ESI+). All reactions were performed in an apparatus with magnetic stirring in an inert atmosphere. Flash column chromatography was performed on a Silica Gel C60 (50–200 μm), Fuji Silysia Chemical Ltd., using an eluent system, as described in the experimental procedures.
procedures. Thin-layer chromatography (TLC) was performed using TLC Silica Gel 60 F254 aluminum sheets (Merck). UV–visible spectra were measured using a Shimadzu RF-1500 spectrophotometer. The fluorescence intensity was recorded on a multi plate reader, BioTek Cytation 3. Fluorescence microscopy imaging was captured using a Keyence BZ-X710 with 20 or 40 objective lenses using the excitation wavelength of 340–380 nm, and the emission collected by the blue channel (435–485 nm) and the green channel (500–550 nm). Luminescence quantum yields of the compounds were recorded on a Hamamatsu Photonics Absolute PL quantum yield spectrometer (C9920-02G). For chemical experiments, general chemicals and solvents, arylboronic acid probes, 2-aminobenzenthiol, 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide, β-galactosidase from Escherichia coli (Grade VI, lyophilized powder, 250–600 units mg⁻¹ protein), doxorubicin, SPIDER-βGal, MeOH, and acetonitrile were purchased from Tokyo Chemical Industries, Fuji fi lm Wako Pure Chemical, and Sigma-Aldrich Co., Ltd. Unless otherwise stated, all materials and reagents were obtained from commercial suppliers and used without further purification. For biological experiments, hydrogen peroxide (H₂O₂) solution (30% w w⁻¹) was purchased from Fuji fi lm Wako Pure Chemical Co., Ltd. H₂O₂ was added by bath application to the medium from a 100 mM aqueous stock (super pure H₂O). The HeLa cells were maintained in Eagle’s minimum essential medium (MEM) purchased from Wako Pure Chemical Industries, Ltd. (Japan), supplemented with 5% fetal bovine serum from Life Technologies (Australia) and 100 unit mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin (Penicillin–Streptomycin mixed, Nacalai Tesque Inc., Japan) at 37 °C in 5% CO₂. The cells were then kept in an incubator (37 °C, 5% CO₂) during the course of the experiments.

**General Synthesis Procedure for 1a–f.** We synthesized arylboronic acids 1a–f according to the literature. 35 2-Formylphenylboronic acid (1.0 mmol) was added to a solution of 2-aminobenzenthiol (107 μL, 1.0 mmol) in MeOH (5.0

**Figure 7.** (A) Images of the HeLa cells cultured with SPIDER-βGal (1.0 μM) after H₂O₂ treatment. (B) Images of the HeLa cells cultured with CBT-β-Gal 4 (12.5 μM) after H₂O₂ treatment. (C) Quantification of the fluorescence in the HeLa cells in the blue channel for the experiment shown in (B). The results are given as mean ± SD.

**Figure 8.** Images of the HeLa cells treated using probe 1a (50 μM), CBT-β-Gal (12.5 μM), and SPIDER-βGal (1.0 μM) after treatment with doxorubicin (100 nM) for 1 day. Images for probe 1a and CBT-β-Gal were captured using the blue channel, whereas the red channel was used for imaging SPIDER-βGal.
mL), and the reaction mixture was stirred at room temperature in open air for 24 h. The organic solvent was then evaporated, and the residue was purified by column chromatography (DCM/MeOH = 9:1); then, the products were recrystallized from dichloromethane (DCM) and methanol (MeOH) to obtain pure products in good yield. The characterization data and experimental procedure are shown in the Supporting Information (SI).

Synthesis of (2-(Benzo[d][1H]-thiazol-2-yl)phenyl)boronic Acid (BTPB). BTPB was prepared according to the general procedure using 2-formylphenylboronic acid (150 mg, 1.0 mmol) and 2-aminobenzenthiol (107 μL, 1.0 mmol) to produce product 1a in a 67% yield. The NMR data of 1a are consistent with the previous report. 29 1H NMR (500 MHz, CD3OD) δ 7.44–7.58 (m, 2H, J = 6.4 Hz, 2H); 13C NMR (125 MHz, CD3OD) δ 121.2, 122.3, 125.5, 125.8, 126.9, 128.6, 130.7, 131.0, 134.6, 135.0, 149.9, 170.5; HRMS (FAB) calcd for C13H10BNO2S [M + H]+ m/z 242.04622, found 242.0622.

Synthesis of Fluorescent Probe CBT-β-Gal (4). 2-(4′-Chloro-2′-hydroxyphenyl)benzothiazole (2). 4-Chlorosalicylaldehyde (470 mg, 3 mmol) was added to a solution of 2-aminobenzenthiol (314 μL, 3.0 mmol) in EtOH (15 mL), and the reaction mixture was stirred at room temperature in open air for 24 h. The organic solvent was then evaporated, and the residue was purified by column chromatography (AcOEt/MeOH = 1:8) to obtain the product 1a in 67% yield. After completion of hydrolysis, the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (AcOEt/MeOH = 1:8) to obtain the product as a white solid (135 mg, 91%). 3H NMR (500 MHz, DMSO-d6) δ 3.37–3.58 (m, 4H), 3.75–3.78 (m, 1H), 3.91–3.96 (m, 1H), 4.67 (d, J = 4.7 Hz, OH), 4.76 (t, J = 5.4 Hz, 1H), 5.01 (d, J = 5.9 Hz, 1H), 5.24 (d, J = 5.9 Hz, 1H), 5.27 (d, J = 7.6 Hz, 1H), 7.28 (dd, J = 2.0, 8.6 Hz, 1H), 7.44–7.47 (m, 2H), 7.54 (dt, J = 1.0, 8.1 Hz, 1H), 8.06 (d, J = 8.1 Hz, 1H), 8.10 (d, J = 7.8 Hz, 1H), 8.44 (d, J = 8.6 Hz, 1H). 13C NMR (125 MHz, DMSO-d6) δ 60.4, 68.1, 70.1, 73.5, 75.9, 101.0, 115.3, 120.8, 121.8, 122.2, 122.5, 124.6, 130.1, 135.8, 136.4, 151.5, 155.3, 161.6; HRMS (FAB) m/z calc for C14H10ClNO2S [M + H]+: 255.0525, found 255.0522.

General Procedure for the UV/Vis Fluorescence Experiments. UV–visible and fluorescence spectra were measured using a Shimadzu RF-1500 spectrophotometer. Probe 1a, HBT, CBT-β-Gal, and CBT were dissolved in DMF to make a stock solution (10 mM). The spectroscopic experiments were performed in solution (DMSO/PBS = 1:1000, v:v). The fluorescence emission spectra were recorded at 470 and 510 nm, with 365 nm as the excitation wavelength.

Selectivity of Probe 1a toward Superoxide Anion (O2−), Hydroxyl Radical (·OH), and Hydrogen peroxide (H2O2). Selectivity experiments were performed according to previous studies. 30,31 H2O2, NaClO, tert-BuOOH, and NaNO2 were prepared for a 4 mM solution in water. To a 96-well plate, the ROS solution (50 μL) and probe 1a (2 μL, 10 mM solution in dimethyl sulfoxide (DMSO)) were added. The final solution volume was adjusted to 200 μL with PBS buffer, resulting in a final concentration of ROS (1 mM, 5 equiv) and probe 1a (200 μM). The reaction of 2,2′-azobis (aminopropane) dihydrochloride (109 mg) and tert-butanol (36 mg) in PBS buffer (10 mL) generated tert- BuOO·* species. ·OH was generated by a Fenton reaction. A mixture of H2O2 (51 μL, 30% in water) and Fe(SO4)2·7H2O (139 mg) in 10 mL of PBS buffer solution was prepared. O2− was from K2O2 (47 mg) in anhydrous DMSO (1 mL). The suspension was diluted to 9 mL of buffer solution, and 4 μL of the solution was immediately transferred to a solution of PBS buffer (194 μL) and probe 1a (2 μL, 10 μM solution in DMSO). The 96-well plate was incubated at 30 °C for 120 min. The fluorescence intensity was recorded at 470 and 510 nm with 365 nm as the excitation wavelength using a multieplate reader, BioTek Cytation 3.

WST-1 Assay. To perform cytotoxicity testing, the HeLa cells (5 × 103 per well) were seeded in a 96-well plate and incubated at 37 °C overnight. The cells were treated with 100 μL of Dulbecco’s modified Eagle’s medium (DMEM) containing varying concentrations of probe 1a or CBT-β-Gal and incubated for 24 h. Subsequently, 10 μL per well of the cell proliferation reagent WST-1 (Roche, Germany) was added to the cells and further incubated at 37 °C for 30 min. The absorbance was measured at 450 nm using a reference wavelength of 600 nm in a TECAN Infinite M200 microplate reader. The relative cell viability in the treated cells was calculated with respect to the untreated cells.

Bioimaging with Probe 1a for Exogenous H2O2 in Living Cells. The HeLa cells were seeded in a 24-well plate and allowed to adhere overnight before treatment. The HeLa cells were incubated in DMEM at 37 °C in a humidified atmosphere containing 5% CO2. After washing the cells with PBS (pH = 7.4), the cells were incubated with 1a (50 μM) for 30 min. Then, the cells were treated with H2O2 (200 or 400 μM) and further incubated for 120 min. After washing the cells
with PBS (pH = 7.4), fluorescence images were obtained using a fluorescence microscope.

**Bioimaging with CBT-β-Gal for β-Galactosidase Activity in Living Cells.** The HeLa cells or Madin–Darby canine kidney cells were plated at a density of 1 × 10^5 cells per well into a 24-well plate. After incubation for 24 h to allow the cells to adhere, the medium was removed, and the cells were washed with PBS. Then, the cells were incubated with CBT-β-Gal (12.5, 25, 50, 100 μM) in DMEM at 37 °C and 5% CO₂ for 24 h. Next, the cells were thoroughly washed three times with PBS and cell fluorescence images were obtained using a fluorescence microscope (see Figure S8).

**Bioimaging with CBT-β-Gal for H₂O₂-Induced β-Galactosidase Activity in Living Cells.** The HeLa cells seeded in a 24-well plate were incubated in DMEM at 37 °C and 5% CO₂ for 24 h. After washing the cells with PBS buffer (pH = 7.4), the cells were treated with H₂O₂ (200 μM) for 2 h. Then, the cells were washed again with PBS and incubated with CBT-β-Gal (12.5 μM) or SPiDER-β-Gal (1.0 μM) for 24 h. Cell imaging was then performed after washing with PBS (pH = 7.4). Cell fluorescence images were obtained using a fluorescence microscope. For SPiDER-βGal (E₄: 525–545 nm, E₆: 605–670 nm).

**Bioimaging of ROS and β-Galactosidase Activity in Dox-Treated Cells.** The HeLa cells seeded in a 24-well plate were incubated for 24 h before treatment. The cells were washed with PBS (pH = 7.4), treated with Dox (100 nM), and further incubated for 24 h. After washing the cells with PBS buffer (pH = 7.4), the cells were incubated with 1a (50 μM), probe 4 (12.5 μM), or SPiDER-βGal (1.0 μM) for 30 min. The cells were washed with PBS buffer (pH = 7.4), and images were obtained using a fluorescence microscope.

**ASSOCIATED CONTENT**

* Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04208.

General experimental procedures and characterization data for the probes; fluorescence spectral changes of probes 1a–e after the addition of H₂O₂; UV–vis absorption and emission spectra; fluorescence images of endogenous ROS in HeLa cells; fluorescence images of endogenous β-galactosidase in HeLa and MDCK cells; NMR spectra of 1a and compounds 2–4 (PDF).

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**Notes**
The authors declare no competing financial interest.

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