Overriding Phthalate Decomposition When Exploring Mycophenolic Acid Intermediates as Selenium-Based ROS Biological Probes

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ABSTRACT: Hypochlorous (OCI⁻) acid is the most well-known bacterial oxidant to be produced by neutrophils. Excess amounts of OCI⁻ can cause various disorders in living systems. Herein, we have designed, synthesized, and characterized two novel organoselenium-based target molecules (Probe-1 and Probe-OCI) based on a synthetic intermediate of mycophenolic acid for the aqueous detection of OCI⁻. Probe 1 has been recently reported (Org. Lett. 2018, 20, 3557–3561); both probes show immediate “turn-on” fluorescence (<1 s) upon the addition of OCI⁻, display an increase in the fluorescence quantum yield (3.7-fold in Probe-1 and 11.6-fold in Probe-OCI), and are completely soluble in aqueous media without the help of any cosolvent. However, a decrease in the “turn-on” intensity with the oxidized version of Probe-1 in cell assays due to the anhydride/phthalate functionality suggests that probe degradation occurs based on hydrolytic action (a probe degradation half-life of ~1500 s at 15 μM Probe-1 and 150 μM OCI). Thus, the change of “anhydride” to “methylamide” begets Probe-OCI, which possesses more stability without sacrificing its water solubility properties and responses at short times. Further studies suggest that Probe-OCI is highly stable within physiological pH (pH = 7.4). Surprisingly, in live cell experiments involving U-2 OS cells and HeLa cells, Probe-OCI accumulated and aggregated in lipid droplets and gives a physiological pH (pH = 7.4). Surprisingly, in live cell experiments involving U-2 OS cells and HeLa cells, Probe-OCI accumulated and aggregated in lipid droplets and gives a “turn-on” fluorescence response. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays confirmed that Probe-OCI is not toxic. Cuvette aggregation studies were also performed (tetrahydrofuran/H₂O) to demonstrate aggregation-induced fluorescence at longer times. Our current hypothesis is that the “turn-on” fluorescence effect is caused by the aggregation-induced emission mechanism available for Probe-OCI. In this case, in tandem, we reanalyzed the Mes-BOD-SePh derivative to compare and contrast cell localization as imaged by confocal microscopy; fluorescence emission occurs in the absence of, or prior to, Se oxidation.

INTRODUCTION

In recent years, many researchers have been focusing on the development and optimization of optical and visualization methods to discern the plethora of important small-molecule analytes such as metal cations, anions, and biologically active neutral or zwitterionic molecules [biothiols and reactive oxygen species/reactive nitrogen species (ROS/RNS)] that are created and exist within the biological system.¹ To achieve this aim, many precise analytical methods have been developed, which involve methods and systems such as potentiometric,² coulometric, colorimetric methods,³ and quantum dot media.¹ Because there is no perfect sensor, the quest to synthesize a soluble medium that is small molecule in nature and also bears other excellent attributes including selectivity, sensitivity, reusability, etc. is still being undertaken. These methods have several drawbacks such as high detection limits [limit of detection (LOD)] and require relatively long times for acquisition of results or extensive procedures, as well as expensive instruments to obtain a quantitative result. On the other hand, fluorescence methods have many advantages: they are specific, sensitive toward specific analytes, easy to handle, and have a high temporal and spatial resolution, thus accommodating the possibilities for both in vitro and in vivo bioimaging.⁵–⁸ Currently, the majority of fluorophores which have been synthesized address problems such as complex or difficult syntheses, large molecular size, and poor solubility; hence, these factors limit the applications of the fluorophore in fluorescence labeling and functionalization. Among the reported organic dyes, maleimide derivatives offer various promising properties, such as high emissivity, large Stokes shifts, water solubility, and ease of modification with many functional groups.⁸b
Through continuous research efforts, more research results have emerged regarding ROS/RNS and its important role in maintaining the physiological and biological processes of the human body. Among other ROS/RNS, hypochlorous acid (OCl⁻) is a highly potent oxidant generated during phagocytosis that serves as a pathogen “killer” within the immune system. OCI⁻ is one of the most biologically important ROS; OCI⁻ is produced by the myeloperoxidase enzyme in leukocytes (macrophages, monocytes, and neutrophils), and the reaction between chloride (Cl⁻) and H₂O₂ gives OCI⁻. The OCI⁻ species, an oxidizing agent of great renown, can undergo reaction with biothiol groups (cysteine, homocysteine, and glutathione) and thioethers (such as methionine) within cells. Because of this action, an excess in concentration of OCI⁻ in biological systems can lead to inflammation-associated tissue damage and a series of life-threatening diseases, such as neurodegenerative disease [i.e., Alzheimer’s disease (AD)], atherosclerotic cardiovascular disease, hepatic ischemia–reperfusion injury, lung injury, and cancer; therefore, the development of new biocompatible classes of molecules that can help for use in highly sensitive and selective ROS fluorescent probes in living systems is vitally important.

To date, a number of fluorescent small molecules have been synthesized and characterized to detect HOCl. However, the real-time detection of HOCl in a biological system remains a challenge. The presence of a cosolvent, low sensitivity, poor photostability and/or chemostability (possibly photobleaching or facile S–E bond cleavage), and long response time, for example, in some cases hinder the actual use in biological systems. Over a period of decades, extensive efforts have been put forth to develop highly selective fluorescent probes for the imaging of ROS at the cellular level. The precise measurement of concentrations of ROS in living systems is a challenging proposition, and chemosensing with excellent selectivity and high sensitivity is the tool in need, which can be employed for the present goal. Many strategies were used to detect HOCl based on its high reactivity; compounds containing thiol, oxime, p-methoxyphenol, and organochalcogen (S, Se, and Te) units have been used. They have displayed highly responsive properties upon oxidation that can be used as HOCl-reactive moieties.

Lipid droplets (LDs) are organelles in cells whose size varies between 20 and 100 nm; LDs conserve and maintain the production of neutral lipids such as seryl esters, triglycerides, and retinyl esters in the cells (eukaryotic and some prokaryotic). LDs can be identified by their hydrophobic central core which is surrounded by a single layer of amphiphilic lipids and proteins, which separate between the aqueous and organic layers. LDs may be found accumulated in the cytoplasm of cells, especially in cells which are responsible for the trafficking and protein maturation. LDs are well-known for their important role in the human immune system such as to modulate the stability of proteins and for exchanging the protein. In addition, LDs are known as a powerhouse for synthesizing molecules important for defense against pathogens such as eicosanoids. Besides that, LDs also help convert overabundant fatty acids into triglycerides in order to separate healthy and toxic fatty acids and keep them housed within relatively inert LDs. Recent studies suggest that lipid oxidation products increase in the brains of AD patients and may involve a significant increase in the concentration of LDs in many diseases characterized by the abnormal concentration of lipid supply and metabolism. LD buildup in cells can occur during the progression of pathologies such as neuropathies, cardiomyopathies, or during liver inflammation caused by the human immunodeficiency virus. The development and progression of several common metabolic diseases such as obesity, type II diabetes, atherosclerosis, and Chanarin-Dorfman syndrome have been associated with the abnormality in neutral lipid storage. Despite their proposed strong relationship to many diseases, our knowledge of LDs is still quite limited.

In continuation of our efforts directed toward the development of molecular probes involving phenyl selenide groups as reactive substituents, we recently reported a novel meso mesitylene-BODIPY system. This system contains a chloro- and phenyl selenide at the 2- and 6-positions, respectively, which can react specifically toward HOCI in vitro and also supply a “turn-on” fluorescence in the presence of LD living cells because of aggregation-induced emission (AIE). The effect of other substituents on the BODIPY system during the detection of HOCI was also investigated.

Even though many application studies have been achieved using phthalate molecules and anhydride derivatives to the best of our knowledge, anhydride and amide phenyl selenide conjugates have never been investigated in such fluorescence studies; here, we pursue selective, water-soluble, and low-molecular-weight detection of HOCI in vitro and demonstrate a “turn-on” response. Because of the susceptibility of phthalates to decomposition, Probe-OCI was investigated in detail despite its apparent vulnerability. Further studies in living cells also demonstrated that Probe-OCI has the ability to accumulate within LDs and act with “turn-on” fluorescence signaling prior to Se oxidation and gives rise to fluorescence because of the AIE effects.

## RESULTS AND DISCUSSION

The syntheses of Probe-1 and Probe-OCI are outlined in Scheme 1 for comparison. The starting materials were synthesized according to a known literature procedure. The diene product underwent reaction with phenylselenyl chloride in the presence of acetonitrile and water and then undergoes a Diels–Alder reaction to form Probe-1 and Probe-OCI in good yield (51 and 62%), respectively. The structures of Probe-1 and Probe-OCI for HOCI detection are shown in Scheme 1.

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**Scheme 1. Synthesis of Probe-1 and Probe-OCI for HOCI**
and Probe-OCl were characterized by spectroscopic techniques [multinuclear nuclear magnetic resonance (NMR)] spectral data, mass spectrometry (MS), and Fourier transform infrared (FT-IR), Figures S4–S18, Supporting Information].

Results from the study of UV–visible spectroscopy of Probe-1 and Probe-OCl are shown in the Supporting Information (Figures S19 and S24). The spectroscopy analysis of Probe-1 and Probe-OCl (15 μM in 10 mM PBS, pH 7.4) is explained in Table 1. Both probes show a "turn-off" emission because of the incorporation of the phenylselenyl group at the 2-position of the probe, which quenches the fluorescence by an intramolecular photoinduced electron-transfer (PET) process that quenches the fluorescence by an intramolecular photoinduced electron-transfer (PET) process and gives a "turn-on" fluorescence after the addition of OCl⁻ (Figure 1).

Both probes inherit identical photophysical properties and selectivity toward OCl⁻. However, Probe-1 is not as stable as Probe-OCl (Figures S19–S23, Supporting Information). We observed that upon the addition of OCl⁻, the emission of Probe-1 gradually decreased over time as observed in the time-dependent experiment (Figure 2A). This could likely occur because of the instability of the anhydride functional group in the aqueous or cellular environment. We suspected that the anhydride motif within Probe-1 undergoes hydrolysis/degradation; thus, decreasing the emission of the resulting molecule proposed to be due to C–O bond rupture and anhydride ring opening and resulting in no loss of the ability for Se to become singly oxidized/oxygenated. Probe-1 and perhaps its depleted form are less likely to aggregate, and our thinking is that the C–O cleavage tendency is only likely to make the tendency for aggregation decrease. On the other hand, the incorporation of methylamide within the skeleton of Probe-OCl affords a much greater stability to Probe-OCl as suggested in the time-dependent experiment (Figure 2B). The stability of Probe-OCl opens the possibility for Probe-OCl to be applied suitably in live cell imaging.

First, we investigated the photophysical properties of Probe-OCl. UV–visible absorption spectra show the maximum absorption at 396 nm. However, upon the addition of OCl⁻ (1.0 equiv) to Probe-OCl, the absorbance peak undergoes a red shift (toward longer wavelength) to 416 nm; whereas after the addition of other ROS/RNS, no significant change in the absorption spectra of Probe-OCl could be observed (Figure S24, Supporting Information). The selectivity of Probe-OCl toward various ROS/RNS (NaOCl, H₂O₂, BuOOH, O₂•−, •OH, •BuO•, NO, and ONOO•) was conducted under physiological conditions of the human body in 10 mM PBS, pH 7.4. At a concentration of 15.0 μM, Probe-OCl showed selectivity toward OCl⁻ (Figure 3A); there is no increase in the fluorescence intensity toward other ROS/RNS even when higher concentrations of other ROS/RNS (20 equiv) were made available. The addition of NaOCl to Probe-OCl gives a

### Table 1. Properties of Probe-1 and Probe-OCl in Solution upon Addition of 10 equiv of OCl⁻ (10 mM PBS, pH 7.4) Incubated for 1.0 min

| Compounds          | λ₁₀₆₄ (nm) | λ₃₉₀ (nm) | ϕ₂ (0.063) | Δλ₂ (nm) |
|--------------------|------------|-----------|------------|----------|
| Probe-1            | 402        | 495       | 0.063      | 93       |
| Probe-1[O]         | 404        | 502       | 0.23       | 98       |
| Probe-OCl          | 396        | 512       | 0.032      | 116      |
| Probe-OCl[O]       | 416        | 523       | 0.37       | 107      |

*aAbsorption wavelength (nm). bEmission wavelength (nm). cFluorescence quantum yield. dStokes shift (nm).*

Figure 1. Comparison of emission spectral changes of Probe-1 (15 μM) and Probe-OCl (15 μM) with various ROS in solution [10 mM phosphate-buffered saline (PBS) pH 7.4] incubated for 5 min, λ₂: 404 nm, λ₃₉₀: 502 nm (Probe-1); λ₂: 416 nm, λ₃₉₀: 523 nm (Probe-OCl). Slit width 3.0 nm/3.0 nm.

Figure 2. Comparison of time-dependent emission spectra of [A] Probe-1 (15 μM) and [B] Probe-OCl (15 μM) with various concentrations of NaOCl in solution (10 mM PBS pH 7.4) incubated for 3600 s, λ₂: 404 nm, λ₃₉₀: 502 nm (Probe-1); λ₂: 416 nm, λ₃₉₀: 523 nm (Probe-OCl). Slit width 3.0 nm/3.0 nm.
Since the presence of even a small aliquot of metal ion solution at high dilution with the reaction and the desired operation of the probe with OCl\(^{-}\) will clearly oxidize selenium to selenoxide in aqueous, as well as in organic solvent, media. We hypothesized that OCl\(^{-}\) will clearly oxidize selenium to selenoxide under sufficient concentrations of analyte. In order to help confirm the reaction mechanism, the characterization of the oxidized species was carried out by high-resolution MS (HRMS) data of Probe-OCl with OCl\(^{-}\).

In order to monitor OCl\(^{-}\) in macrophage cells, Probe-OCl should remain stable in a slightly acidic environment without eliciting any fluorescence response. Hence, we tested the response of Probe-OCl toward OCl\(^{-}\) at various pH levels (Figure 4). The results reveal that, as expected, the pH value of the solution has an influence on the fluorescence response to [OCl\(^{-}\)]. As shown in Figure 3, the fluorescence intensity at 523 nm of the Probe-OCl response to OCl\(^{-}\) was gradually enhanced when the pH increased in the range of 6.0–7.0; it reached the maximum intensity in the range of 7.4–11.0. However, in the pH range of 4.0–5.0, the probe did not respond to OCl\(^{-}\); we did not observe any fluorescence enhancement in this pH region (4–5) because of the pH effect toward Probe-OCl detection. The trend of fluorescence intensity changes of Probe-OCl at 523 nm demonstrates that the probe can tolerate a slightly acidic environment; because of this, it is suitable to detect OCl\(^{-}\) in living media and cellular subcompartments such as macrophage cells.

Titration of Probe-OCl with the increase of the concentrations of NaOCl (0–150 \(\mu\)M) was carried out; the experiments supported that the emission intensity of Probe-OCl proportionally increases upon the addition of NaOCl from 0 to 10 equiv (Figure 3B). The fluorescence of Probe-OCl reached emission saturation after the addition of 9 equiv of NaOCl. Further study showed that the LOD of the probe was determined to be 90 nM (Figure S27, Supporting Information).

The time-dependent study of Probe-OCl and Probe-1 was carried out simultaneously with the addition of 10 equiv of NaOCl. Probe-OCl shows a very fast response upon the addition of NaOCl and gives a strong green fluorescence just after the addition of NaOCl (Figure 5). On the other hand, we observed a rapid decrease in the emission intensity with Probe-1. The time-dependent experiments of Probe-OCl indicate that Probe-OCl reacts very fast with OCl\(^{-}\); this result opens up the possibility of Probe-OCl to be used as a real-time sensing agent in the cellular level.

It has been known that OCl\(^{-}\) has an ability to oxidize selenium to selenoxide in aqueous, as well as in organic solvent, media. We hypothesized that OCl\(^{-}\) will clearly oxidize selenium to selenoxide under sufficient concentrations of analyte. In order to help confirm the reaction mechanism, the characterization of the oxidized species was carried out by high-resolution MS (HRMS) data of Probe-OCl with OCl\(^{-}\).
Selenoxide formation has occurred after the addition of OCl to the intensity of the probe. These HRMS data support that blocking of the PET process and an increase in the emission of the phenyl selenide donor moiety to the formation of the selenoxide blocks electron transfer from the [M + Na+] (Figure S16, Supporting Information). In addition, addition of 10 equiv of L-cysteine after 5 min in solution (10 mM PBS pH 7.4) showed the ability to revert the selenium oxide species (R2SeO) to its original reduced state (R2Se). The result (Figure 6) showed a significant decrease in the fluorescence intensity with glutathione, L-cysteine, and homocysteine. In the case of glutathione and homocysteine addition, oxidized Probe-OCl gives a “turn-off” fluorescence signal after 5 h of incubation time involving a screening that simulates the sequence of several redox cycles (Figure S33, Supporting Information).

To confirm the nature of the photomechanism at play, density functional theory (DFT) geometry optimization calculations were performed. Optimized structures were estimated by DFT calculations using the Gaussian 09 program. The B3LYP functional with a 6-31g* basis set was used, and the 6-311g* basis set was used only for Se; all calculations were performed in the gas phase (Figure S28, Supporting Information). As observed by considering the geometry of the optimized structure of Probe-OCl and Probe-OCl[O], the case of these two kinds of molecular orbital electron density maps is surprising; they seem almost the same and are without the involvement of the selenyl position. Also, the difference in energy between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of DFT-optimized geometries of probe and oxidized probe is almost the same. Differences of electronic distributions were observed in the case of HOMO = 2, HOMO, and LUMO + 1 orbitals. In parts of electronic distributions for HOMO = 2, LUMO + 1, the phenylselenium moiety does not possess electronic distribution density, and in the HOMO segment, all of the electronic distributions involved the phenylselenium moiety. Therefore, HOMO-LUMO electronic distributions changed from being directly on the phenylselenium moiety to being on the center of the naphthalimide moiety. On the other hand, the electron transfer from phenylselenium to the naphthalimide core of the chemically oxidized probe could have occurred more effectively than for the probe because the electronic distributions seen in the HOMO = 2, HOMO, LUMO + 2 level overlapped with phenylselenium from the naphthalimide core (Figure S29, Supporting Information). From these results, the fluorescence “turn-on” event could be explained by a blockage of the PET that would ordinarily exist between phenylselenium and the naphthalimide core occurring from the oxidation of the selenium center (Table S3, Supporting Information).

To further demonstrate the applicability of Probe-OCl, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed. Probe-OCl was found to be nontoxic to cells and did not interfere with cell proliferation (Figures S31 and S32, Supporting Information). To examine prospective biological applications of Probe-OCl, U-2 OS (Figure 7) and HeLa cells (Figure S32, Supporting Information) were chosen for live cell imaging. DRAQ5 was used to costain the nucleus of the cells; 4’,6-diamidino-2-phenylindole and Alexa-488 were used because of the similar range of excitation wavelength with Probe-OCl and MES-BOD-SePh. Then, U-2 OS cells were treated with Probe-OCl, followed by a 10 min incubation period, and the cells were expected to exhibit low fluorescence based on the presence of the synthetic molecule. Surprisingly, a strong fluorescent dot pattern was observed (Figure 7A). Then, we compared the Se results with those from our previous work using BODIPY mesitylene functionalized at the so-called meso-position (MES-BOD-SePh). We conclude that the fluorescence observed could be caused by the aggregation of the probe within a LD, as the images showed a similar pattern and overlapped with the previous developed MES-BOD-SePh.

![Figure 5](image-url) Comparative time-dependent emission spectra of Probe-OCl (15 μM) and Probe-1 (15 μM) with the addition of 10.0 equiv of NaOCl in the 10 mM PBS pH 7.4, followed by incubation for 3000 s; λ<sub>ex</sub>: 404 nm, λ<sub>em</sub>: 502 nm (Probe-1) λ<sub>ex</sub>: 416 nm, λ<sub>em</sub>: 523 nm (Probe-OCl). Slit width 3.0 nm/3.0 nm.

![Figure 6](image-url) Reversibility of the oxidized Probe-OCl (15 μM) with the addition of 10 equiv of L-cysteine after 5 min in solution (10 mM PBS pH 7.4); λ<sub>ex</sub>: 416 nm, λ<sub>em</sub>: 523 nm. Width 3.0 nm/3.0 nm.
which selectively targets the LDs (Figure 7B). Furthermore, to confirm that our Probe-OCI selectively targets LDs, both probes were counterincubated and observed to overlap in their signals and fluorescence characteristics (Figure 7C).

The AIE feature of Probe-OCI was investigated in a tetrahydrofuran (THF)/water mixture, by measuring the increase of the fluorescence emission spectrum of Probe-OCI (Figure 7). Probe-OCI exhibited weak emission in THF; there is no increase in the emission intensity up to a 9:1 (v/v) THF/water mixture. Surprisingly, we observed a significant increase in the emission intensity in a THF/water mixture when a ratio of 4:6 is used (Figure 8). The fluorescence intensity decreased dramatically and reached the lowest intensity measured when the water fraction reached ~80%. However, when the water fraction reached ~90%, the fluorescence intensity increased. These optical changes are attributed to twisted intramolecular charge-transfer effects arising from increased solvent polarity as seen through a solvent system that is gradually increasing in its portion of water.67 The addition of water can effectively influence the present conditions of Probe-OCI; therefore, Probe-OCI possesses AIE properties.

■ CONCLUSIONS

In summary, two fluorescent probes, Probe-1 and Probe-OCI, were scrutinized and compared in new cellular media and directly prepared and characterized based on a synthetic intermediate of mycophenolic acid for the specific detection of hypochlorous acid (HOCl). Both probes showed sensitive, selective, and fast responses to HOCl over other ROS/RNS because of the oxidation of selenide to selenoxide. Both probes showed immediate “turn-on” fluorescence responses (<1 s) upon the addition of OCI− and showed an increase in their quantum yield (3.8-fold in Probe-1 and 11-fold in Probe-OCI); they are completely soluble in aqueous media without the help of any cosolvent. Further studies showed that Probe-1 is unstable in a water environment at longer times and undergoes what could be categorized as fluorescence photobleaching. Therefore, we incorporated methylmaleimide within Probe-OCI to help increase the stability of the compounds. The superiority of Probe-OCI includes high sensitivity,
specifity, and rapid “turn-on” fluorescence response for HOCI under physiological pH. Surprisingly, when Probe-OCI was studied in living cells (U-2 OS cells and HeLa cells), MTT assays confirmed that Probe-OCI is not toxic. Probe-OCI undergoes AIE in LDs. We reanalyzed the Mes-BOD-SePh derivative for a comparative localization of probes. Probe-OCI was reported to be a probe for LDs as seen in cuvette aggregation and induced fluorescence based on the addition of OCI− but lacks stability-based hydrolytic action of water (probe degradation with a half-life of ~1500 s at 15 μM Probe-1 and 150 μM OCI−). Thus, the change of “anhydride” to “methylamide”−an interesting substitution to explore in medicinal chemistry−led us to investigate Probe-OCI. This system is more appropriate, and this phenomenon helped make determinations within the range of physiological pH and based on associated attributes such as stability than compared with Probe-1.

## EXPERIMENTAL SECTION

### General Considerations.
All chemicals were used without further purification and purchased from commercial sources (Aldrich, Tokyo Chemical Industry). 1H, 13C, 77Se NMR spectra were acquired using a Bruker Avance 400 and Agilent-NMR-vnmrs 600 MHz spectrometer. Tetramethylsilane (TMS) and dimethyl selenide were used as external standards.

ESI-MS was performed on a Bruker micrOTOF-QII by the research support sta

A solution of triethylamine hydrochloride was formed, precipitated, and then poured over crushed ice. The precipitated product was filtered off, washed by the addition of ice water, dried, and concentrated. The residue was purified by silica gel flash column chromatography using n-hexane/EtOAc (3:2) as an eluent to afford Probe-1 (0.260 g, 51%) as a yellow solid. 1H NMR (600 MHz, CDCl3): δ 7.64−7.61 (m, 2H, H12, H16), 7.52−7.49 (m, 1H, H14), 7.47−7.44 (m, 2H, H13, H15), 6.74 (s, 1H, H6), 2.63 (s, 3H, H10); 13C NMR (150 MHz, CDCl3): 164.2 (C1), 162.4 (C8), 153.5 (C9), 152.2 (C13), 136.7 (C12,16), 131.1 (C10), 130.4 (C13,15), 130.1 (C12), 127.3 (C7), 125.9 (C1), 122.7 (C6), 117.1 (C1), 15.0 (C5); 77Se NMR (76.5 MHz, CDCl3): 430.68; IR (CHCl3) νmax: 3472, 3020, 2928, 1835, 1757, 1622, 1465, 1440, 1405, 1369, 1293, 1215, 1166, 1039, 980, 908, 756 cm−1; mp = 189−192 °C; MS-EI m/z: calcd for C15H10O4Se + Na, 356.9642; found, 356.9622.

### Synthesis of Probe-OCI (7-Hydroxy-2,4-dimethyl-5-(phenylselanyl)isoindoline-1,3-dione).
A solution of triethylamine (0.472 mL, 3.40 mmol) in acetonitrile (5 mL) was added dropwise to a solution of 5-methyl-4-(phenylselanyl)furan-2(5H)-one (0.500 mg, 2.80 mmol) and trimethylacetyl chloride (0.417 mL, 3.40 mmol) in acetonitrile (15 mL), and the mixture was stirred at 60 °C for 14 h. The precipitate of triethylamine hydrochloride was formed and filtered off. The filtrate was washed with 10% sodium carbonate, dried over MgSO4, and concentrated. The residue was concentrated and filtered through silica gel using a petroleum ether/EtOAc (4:1) solvent combination to afford the diene as a colorless oil. This was used directly for the next reaction. The above-mentioned diene (0.600 g, 1.53 mmol) and N-methylmaleimide (0.150 g, 1.53 mmol) were dissolved in diethyl ether (3 mL) under N2 and stirred overnight. Concentrated H2SO4 (1.5 mL) was added slowly to this resulting sticky liquid product. The cream-colored mixture was stirred for 5 min and then poured over crushed ice. The precipitated product was filtered off, washed by the addition of ice water, dried, and concentrated. The residue was purified by silica gel flash column chromatography using n-hexane/EtOAc (3:2) as an eluent to afford Probe-OCI (0.597 g, 62%) as a yellow solid. 1H NMR (400 MHz, CDCl3): δ 7.58−7.56 (m, 2H, H12, H16), 7.48−7.32 (m, 3H, H13, H15, H16), 6.62 (s, 1H, H6), 3.06 (s, 3H, H12), 2.57 (s, 3H, H10); 13C NMR (100 MHz, CDCl3): 169.8 (C16), 168.6 (C8), 152.5 (C7), 147.9 (C9), 136.2 (C12,16), 130.1 (C13,15), 129.4 (C10), 129.3 (C14), 127.8 (C11), 126.8 (C13), 121.9 (C9), 112.3 (C7), 23.5 (C5), 15.0 (C5); 77Se NMR (76.5 MHz, CDCl3): 416.81; IR (CHCl3) νmax: 3434, 3201, 2933, 1758, 1694, 1615, 1578, 1439, 1385, 1285, 1252, 1204, 1177, 1160, 1086, 1066, 1005, 928, 871, 757 cm−1; mp = 135−140 °C; MS-EI m/z: calcd for C16H10NO3Se + H, 348.0119; found, 348.0119.

### Preparation of Samples.
Stock solutions (10 mM) of Probe-1 and Probe-OCI were prepared in dimethyl sulfoxide.

The test solution was prepared by the addition of 15 μM Probe-1 and Probe-OCI to 3 mL of PBS (10 mM, pH 7.4). After that, analytes were added, and a vortex mixer was used to make solutions homogeneous and ready for measurements.
**Measurement of Quantum Yield.** The fluorescence quantum yield of **Probe-1** and **Probe-OCl** before and after the reaction with **OCI**<sup>-</sup> was measured with fluorescein as a reference (0.10 M NaOH). The quantum yield was calculated with a comparative method according to the equation

\[
\Phi_Q = \frac{Q_R (m/n_R)}{m/n_R}
\]

where \(Q_R\) is the fluorescence quantum yield of fluorescein, \(m\) is the slope of the line obtained from the plot of the integrated fluorescence intensity versus absorbance, and \(n\) is the refractive index of the solvent. Subscript \(R\) refers to the reference (fluorescein).

**Determination of LOD.** LOD of probes was calculated from fluorescence titration measurements and blank measurements. To obtain the value for the slope, the values of fluorescence titration measurement intensity for **Probe-1** (502 nm) and **Probe-OCl** (523 nm) were used, respectively. Therefore, the detection limit was calculated with the following equation:

\[
\text{Detection limit} = 3\sigma/k
\]

where \(\sigma\) is the standard deviation of 10 blank measurements and \(k\) is the absolute value of the slope between the fluorescence intensity versus the HOCl concentration.

**DFT Calculations.** The molecular structures of **Probe-OCl** and **Probe-OCl**<sup>[O]</sup> and their HOMO–LUMO levels were calculated by DFT calculations by Gaussian 09 software (B3LYP method with 6-31g* basis set for Se only and 6-31g*).

**Cell Maintenance and Live Cell Imaging.** HeLa and U-2 OS cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) or RPMI 1640 (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1% penicillin/streptomycin (100 U/mL, Gibco). The HeLa cells were seeded at a density of 0.6 × 10<sup>5</sup> cells on sterilized μ-slides (ibidi, München, Germany). After 24 h, cells were washed with Dulbecco’s PBS (D-PBS) two times. Next, 25 μM **Probe-OCl** and 25 μM MES-BOD-SePh were added to the cells, and the cells were incubated for 10 min at 37 °C and washed with D-PBS two times. Live cell images were acquired on a Zeiss LSM 780 (Carl Zeiss, Jena, Germany) laser scanning confocal microscope, and 63X objective lens were used. The excitation wavelengths were 405 and 488 nm, and the detection wavelengths were 410–495 and 500–563 nm.

**Cell Viability Assays.** The proliferation of HeLa cells was determined by the MTT assay (Sigma-Aldrich) using an established procedure. Briefly, the HeLa cells were seeded at a density of 1 × 10<sup>4</sup> cells into a 96-well plate (n = 4). Next, the cells were pretreated with **Probe-OCl** at various concentrations (10, 20, 30, 40, and 50 μM) for 1–6 h. After incubation, the proliferation reagent MTT (0.5 μg/mL) was added to each well, and the reagent-applied cells were incubated for 3 h at 37 °C and under 5% CO<sub>2</sub>. Absorbances were measured using a Varioskan Flash (Thermo Scientific) at 540 nm, and the reference wavelength was 690 nm.

**Author Contributions**

M.B.H., T.Y., and K.J.L. contributed equally.

**Notes**

The authors declare no competing financial interest.

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