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HKOCl-2 Series of Green BODIPY-Based Fluorescent Probes for Hypochlorous Acid Detection and Imaging in Live Cells

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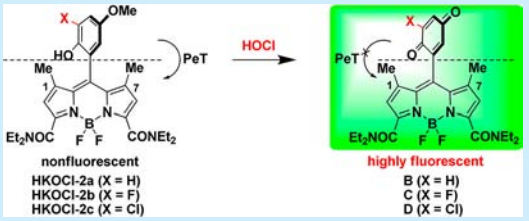
Supporting Information

ABSTRACT: A **HKOCl-2** series of new fluorescent probes for hypochlorous acid (HOCl) detection in live cells is reported. The probes exhibit excellent selectivity, sensitivity, and chemostability toward HOCl. In particular, **HKOCl-2b** rapidly and selectively detects endogenous HOCl in both human and mouse macrophages. These probes could therefore serve as promising discovery tools to help elucidate biological functions of HOCl.



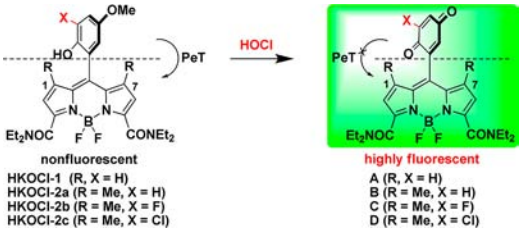
Reactive oxygen/nitrogen species (ROS/RNS) such as superoxide, hydrogen peroxide, nitric oxide, hydroxyl radical, hypochlorous acid (HOCl), and peroxynitrite are involved in fundamental cellular processes including inflammation, aging, cancer, and neurodegeneration.[1] Over the past decade, extensive efforts have been invested in the development of selective fluorescent probes for imaging cellular ROS/RNS.[2,3] Among ROS/RNS, HOCl is a highly potent oxidant and exhibits remarkable reactivity toward various protein side chains and peptide bonds,[4] which in part accounts for its role as an important microbicidal effector in innate immunity.[5−7] Endogenous HOCl can be generated in a regulated manner from hydrogen peroxide and chloride by the enzyme myeloperoxidase (MPO).[8] However, abnormal HOCl production derived from phagocytes is linked to many diseases including cardiovascular diseases,[9,10] inflammatory diseases,[4,11] neurodegenerative diseases,[12] cystic fibrosis,[13] kidney diseases,[14] and certain cancers.[15]

Although many fluorescent probes for HOCl detection have been reported in recent years,[16−46] only a fraction of them showed practical utility in further biological investigations due to common problems in selectivity, sensitivity, chemostability, and/or photostability.

Previously, our group reported a green fluorescent probe HKOCl-1 for HOCl with high selectivity and sensitivity.[21] However, its fluorescent product **A** can be further oxidized by HOCl to give compounds of weaker fluorescence, resulting in compromised fluorescence enhancement. One possible decomposition pathway is the epoxidation of the quinone moiety, as we found that 4-methoxyphenol was indeed oxidized by 2 equiv of HOCl to give 2,3-epoxy-1,4-benzoquinone in 65% yield (see the Supporting Information (SI)). To overcome this limitation, we have developed the **HKOCl-2** series of fluorescent probes with excellent selectivity, sensitivity, and chemostability for HOCl detection and imaging in live cells.

In order to prevent the overoxidation of the quinone product **A**, generated by HKOCl-1 reaction with HOCl, we have designed **HKOCl-2a** with two methyl groups added to the 1 and 7 positions of the BODIPY core of HKOCl-1 to increase the steric hindrance around 4-methoxyphenol (Scheme 1). One additional advantage is that the quantum yield of the fluorescent product **B** is expected to be higher than that of **A** because the rotational degree of freedom of the quinone moiety is further restricted by the two added methyl groups.[47] Moreover, *ortho*-halogen substituted analogues **HKOCl-2b** and **HKOCl-2c** have been designed to further block oxidation of the quinone products **C** and **D**, which have been confirmed by ESI-MS measurement (see the SI). Furthermore, according to the HOMO energy calculations (Scheme S1), the extra substituents on **HKOCl-2a−c** should not change the mechanism for fluorescence turn-on upon oxidation by HOCl, that is, **HKOCl-2a−c** are expected to be nonfluorescent due to the photoelectron transfer (PeT) process whereas oxidation products **B−D** should be fluorescent due to suppression of PeT.

Scheme 1. Structures and Design of HKOCl-1 and an HKOCl-2 Series of New Fluorescent Probes for HOCl Detection



The designed **HKOCl-2** series of probes (**HKOCl-2a**, **HKOCl-2b**, and **HKOCl-2c**) has been prepared from

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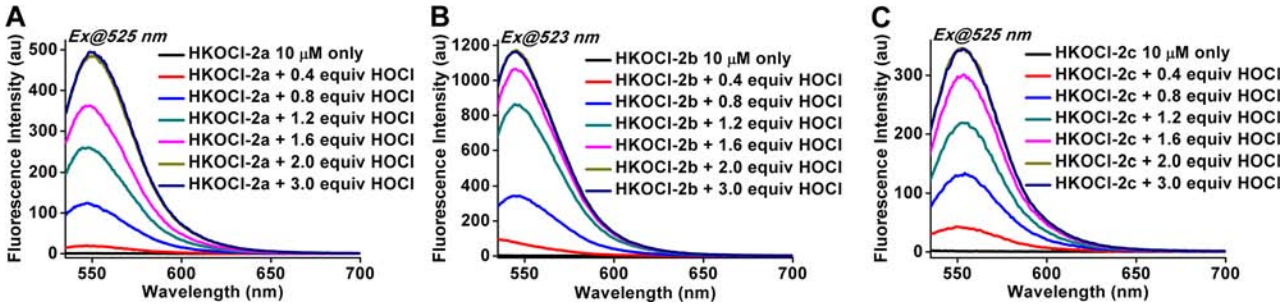

Figure 1. Fluorescence emission spectra of the fluorescent probes (A) **HKOCl-2a** (10 μM), (B) **HKOCl-2b** (10 μM), and (C) **HKOCl-2c** (10 μM) in 0.1 M phosphate buffer (0.1% DMF, pH 7.4) upon treatment of increasing amounts (0−3 equiv) of HOCl for 30 min.

corresponding aldehydes and methylated pyrrole according to Scheme S3. We first investigated the reactivity of those probes toward HOCl in 0.1 M potassium phosphate buffer at pH 7.4. As expected, in the absence of HOCl, the probes were nonfluorescent; on exposure to HOCl, a very rapid fluorescence increase was observed with emission maxima at around 545 nm (Figure 1), and the fluorescence intensity reached a plateau within 15 min (Figure 2).

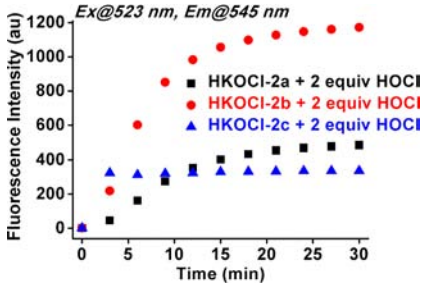

Figure 2. Time course of fluorescence intensity of probes **HKOCl-2a−c** upon treatment with 2 equiv of HOCl over a period of 30 min. Probes were dissolved in 0.1 M potassium phosphate buffer at pH 7.4 to a final concentration of 10 μM (containing 0.1% DMF).

As shown in Figure 3, the fluorescence intensity of all three probes at around 550 nm became saturated when 2 equiv of

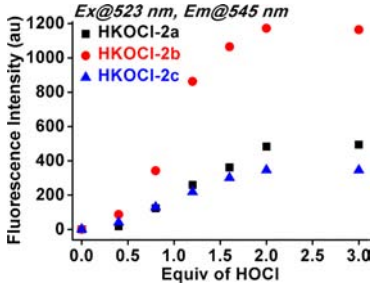

Figure 3. Dose response of fluorescence emission intensity of probes **HKOCl-2a−c** toward HOCl (0−3 equiv). Probes were dissolved in 0.1 M potassium phosphate buffer at pH 7.4 to a final concentration of 10 μM (containing 0.1% DMF). The fluorescence spectra were recorded at 30 min after addition of HOCl.

HOCl were added and almost no change in fluorescence intensity was observed upon treatment with 10 equiv of HOCl (Figure S2), demonstrating the chemostability of fluorescent products toward HOCl. Among the three probes (Φ < 0.002), **HKOCl-2b** gave the strongest turn-on fluorescence response toward HOCl (Figure 3). Although the dose response was not linear, the detection limit (S/N = 3) was estimated to be 42, 18, and 37 nM for **HKOCl-2a**, **HKOCl-2b**, and **HKOCl-2c**, respectively. Overall, the above-mentioned results confirmed the excellent chemostability of the fluorescent products of the **HKOCl-2** series of fluorescent probes upon treatment with HOCl.

Next, the selectivity of the **HKOCl-2** series of fluorescent probes toward various analytes has been tested. As shown in Figure 4, over 900-fold enhancement in fluorescence intensity was observed with **HKOCl-2b** (10 μM) in the presence of 2 equiv of HOCl (final concentration 20 μM), whereas 10 equiv of other ROS/RNS including H_2O_2, $^1O_2$, ROO$^\bullet$, TBHP, $^\bullet$NO, $O_2^{\bullet-}$, $^\bullet$OH, and $ONOO^-$ (2 equiv) induced negligible fluorescence increase after 30 min (Figure 4). **HKOCl-2a** and **HKOCl-2c** have also been found to be highly selective toward HOCl (Figure 4). According to literature, under physiological or pathophysiological conditions, the steady-state $ONOO^-$ concentrations are estimated to be in the nanomolar range,[48] while concentrations of HOCl are estimated to be in the micromolar range.[49] As such, the peroxynitrite concentration is unlikely to surpass that of HOCl in most cases, to become an important interfering species in HOCl detection. Collectively, the excellent selectivity of the **HKOCl-2** series of fluorescent probes, especially **HKOCl-2b**, toward HOCl over other ROS/RNS, confirms our design principle.

As pH is an important factor in cellular HOCl detection, the pH-dependence of our probe **HKOCl-2b** was validated in buffers prepared in the biologically relevant pH range (3−12). The fluorescence increase of **HKOCl-2b** toward HOCl was significant across a wide range of pH (4−10; Figure 5). Thus, this result not only demonstrates the excellent pH stability of **HKOCl-2b** but also illustrates that our probe **HKOCl-2b** is likely to be biocompatible.

Based on its superior performance in selectivity and sensitivity in chemical characterization, **HKOCl-2b** was chosen among the three probes for functional elaboration in live cell imaging. To induce endogenous HOCl, RAW264.7 mouse macrophages were coincubated with **HKOCl-2b** (10 μM) and the PKC activator PMA (phorbol myristate acetate; 200 ng/mL) or Toll-like receptor-2 (TLR2) ligand zymosan (*Saccharomyces cerevisiae*; 10 μg/mL) for 30 min, followed by confocal imaging (Figure 6). The NOX (NADPH oxidase) specific peptide inhibitor gp91ds-tat (2.5 μM) was added to block superoxide production. As anticipated, both PMA and zymosan significantly increased HOCl production in activated macrophages (Figure 6), with a very well-defined cytoplasmic fluorescence distribution. Inhibition of superoxide generation by gp91ds-tat potently reduced the **HKOCl-2b** fluorescence signal to near basal level, confirming that the observed ROS

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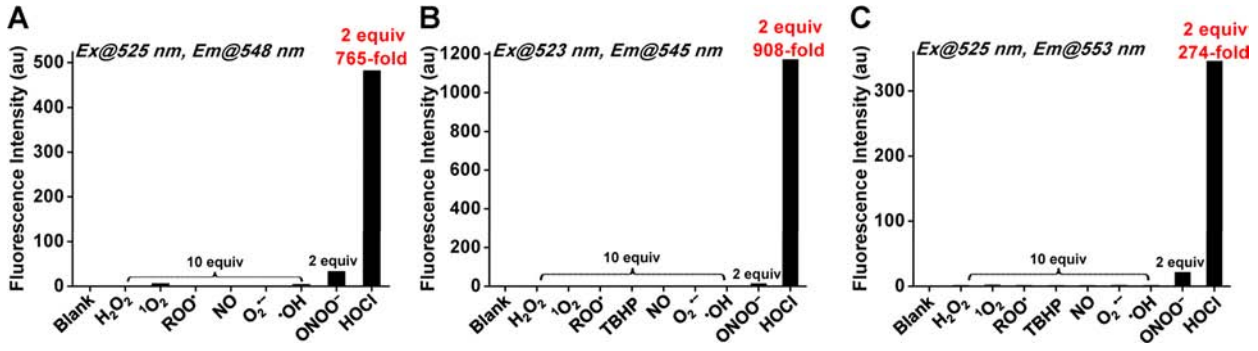

Figure 4. Test of selectivity of fluorescent probes (A) **HKOCl-2a** (10 μM), (B) **HKOCl-2b** (10 μM), and (C) **HKOCl-2c** (10 μM) in 0.1 M phosphate buffer (0.1% DMF, pH 7.4) toward various ROS and RNS. Fluorescence spectra were recorded at 30 min.

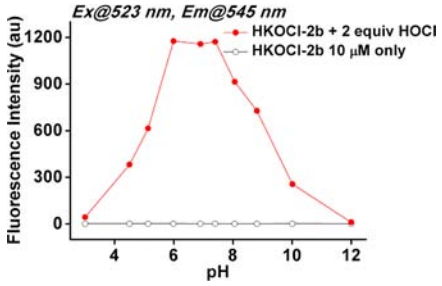

Figure 5. Fluorescence response of **HKOCl-2b** (10 μM) in 0.1 M potassium phosphate buffer across a wide pH range (3−12) toward HOCl (20 μM) was measured.

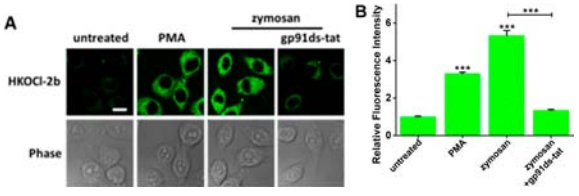

Figure 6. Detection of endogenous HOCl in activated RAW264.7 mouse macrophages. (A) Cells were incubated with **HKOCl-2b** (10 μM) in the presence or absence of PMA (200 ng/mL), zymosan (10 μg/mL), or zymosan plus gp91ds-tat (2.5 μM) for 30 min, before confocal imaging. (B) Quantitative analysis of endogenous HOCl production by measuring the mean fluorescence of cells under the same pharmacological treatments as in (A) (n = 20 cells). ***, p < 0.001. Scale bar represents 10 μm.

production was enzymatically controlled. The results suggest that **HKOCl-2b** is highly sensitive and rapid in its turn-on response in live cells.

Next, we attempted to visualize endogenous HOCl in human THP-1 monocytic macrophages, which have not been validated for HOCl production despite being known to express MPO at low to moderate levels.[50] THP-1 cells were first differentiated into macrophages as previously described.[51] Cells were then coincubated with **HKOCl-2b** (10 μM) and PMA (200 ng/mL) in the presence or absence of the PKC inhibitor Gö6983 (100 nM) or NOX inhibitor DPI (100 nM) for 30 min, followed by confocal imaging (Figure 7). In the presence of PMA, the **HKOCl-2b** fluorescence response was significantly enhanced, while both Gö6983 and DPI could efficiently reduce this increased fluorescence to a level comparable to that of the unstimulated control. These results suggest that endogenous HOCl production in differentiated human macrophages is coupled to the PKC/NOX signaling pathway and can be

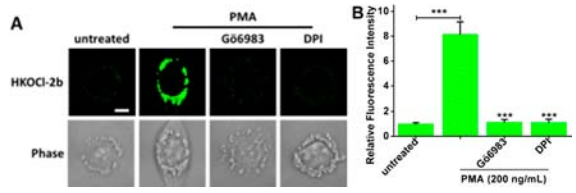

Figure 7. Detection of endogenous HOCl in differentiated THP-1 human macrophages. (A) Cells were incubated with **HKOCl-2b** (10 μM) in the presence or absence of PMA (200 ng/mL), PMA plus Gö6983 (100 nM), or PMA plus DPI (100 nM) for 30 min, before confocal imaging. (B) Quantitative analysis of endogenous HOCl production by measuring the mean fluorescence of cells under the same pharmacological treatments as in (A) (n = 6−13 cells). ***, p < 0.001. Scale bar represents 10 μm.

reproducibly manipulated by established pharmacological agents.

Lastly, to further validate the selectivity of **HKOCl-2b** toward HOCl in cells, we used yeast zymosan as a stimulant to mimic the phagocyte oxidative burst in response to fungal infection. Differentiated THP-1 cells were coincubated with **HKOCl-2b** (10 μM) and zymosan (10 μg/mL) in the presence or absence of the specific HOCl scavenger taurine (5 mM) or MPO inhibitor ABAH (4-aminobenzoic acid hydrazide; 100 μM) for 30 min, followed by confocal imaging (Figure 8). Zymosan rapidly triggered **HKOCl-2b** fluorescence enhancement comparable to that in PMA treatment. As expected, taurine and ABAH greatly attenuated the **HKOCl-2b** fluorescence signal to basal levels, again suggesting the high selectivity of the probe toward HOCl.

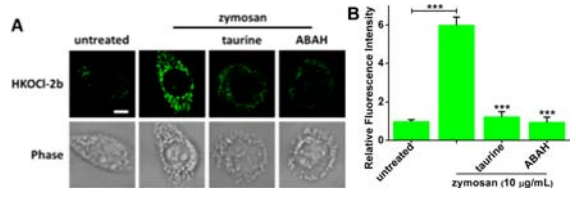

Figure 8. Test on selectivity of **HKOCl-2b** toward endogenous HOCl in differentiated THP-1 human macrophages. (A) Cells were incubated with **HKOCl-2b** (10 μM) in the presence or absence of zymosan (10 μg/mL), zymosan plus taurine (5 mM), or zymosan plus ABAH (100 μM) for 30 min, before confocal imaging. (B) Quantitative analysis of endogenous HOCl production by measuring the mean fluorescence of cells under the same pharmacological treatments as in (A) (n = 6−13 cells). ***, p < 0.001. Scale bar represents 10 μm.

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In conclusion, we have successfully designed, synthesized, and characterized several fluorescent probes for HOCl detection. In particular, compared to our pervious probe HKOCl-1, the new probe **HKOCl-2b** exhibits excellent selectivity, sensitivity, and chemostability. In live cell imaging, **HKOCl-2b** gave rapid turn-on fluorescence in response to various established HOCl induction stimulants including PMA and zymosan in both mouse and human macrophages, in a highly selective and sensitive manner. Overall, our probes possess attractive properties as molecular tools for endogenous HOCl detection in live cells and could be exploited more productively in biological investigations.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis, characterization, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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