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Design and characterization of 3-Azidothalidomide as a selective hydrogen sulfide probe

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Abstract

Hydrogen sulfide (H_2S) has been recently recognized as an important signaling molecule in biological systems. Herein, we report the development of a fluorescence turn-on probe based on the structure of pomalidomide, a FDA approved drug for the treatment of multiple myeloma. Various characterizations demonstrated high selectivity and sensitivity of this probe towards H_2S . Furthermore, the application of this probe to detect H_2S in living cells was confirmed by flow cytometry and fluorescence imaging studies.

Keywords

Pomalidomide; hydrogen sulfide; azide reduction; fluorescent probe

Hydrogen sulfide (H_2S), one of the reactive sulfur species, is a colorless gas and can be produced enzymatically from the metabolism of cysteine by cystathionine- β -synthase, cystathionine- γ -lyase, or 3-mercaptopyruvate sulfur transferase in various tissues.^{1–4} Accumulating evidence has recently indicated that H_2S , like nitric oxide and carbon monoxide, is a gaseous signaling molecule and plays important roles in many physiological functions,^{5–7} such as neuromodulation^{8,9} and inflammation.^{10,11} Changes in H_2S levels have also been implicated in several pathological conditions such as Alzheimer's disease, hypertension, and diabetes.^{12,13} Along with the discovery of its diverse functions under pathophysiological conditions, methods of selective and sensitive detection of H_2S have also

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been extensively studied.^{14–16} Among the methods developed, fluorescent probes have attracted extensive attention because of their high sensitivity and spatiotemporal resolution capacities with potential applications in biological systems. Recently significant progress has been made in the development of fluorescent probes with improved sensitivity, and these probes are mainly based on H₂S-dependent reactions, namely H₂S-mediated reduction of azides^{17–23}, nucleophilic addition²⁴, and copper sulfide precipitation^{25,26} in order to achieve fluorescence off-on responses.

During previous work on thalidomide analog development, we noted that 3-aminothalidomide (1, Fig. 1), also known as pomalidomide (approved for multiple myeloma treatment by FDA in 2013), is a fluorescent molecule with good water solubility. Not like many other fluorescent molecules, extensive toxicity studies have been done and documented for pomalidomide and it is safe for in vivo application. Interestingly, 3-aminophthalimide, a fragment of 1, has been reported as a fluorophore²⁷ and some of its analogs and/or conjugates have been studied for ion detection²⁸ or exploring ligand protein binding interactions.²⁹ Collectively, this may indicate that 1 could be a biologically compatible fluorophore. Taking advantage of the known reduction of azides by H₂S and easy synthesis with large modification potential, herein we attempted to replace the 3-NH₂ of 1 with 3-N₃ and to characterize this azide analog as a fluorescence turn-on probe for H₂S (2, Fig. 1). Furthermore, 4-azidothalidomide (4, Fig. 1) has been reported as a photo affinity probe to identify the potential targets of thalidomide.³⁰ Considering the possibility that 4-aminothalidomide (3, Fig. 1) may emit fluorescence, we also prepared compound 4 for comparison.

After chemical synthesis of these compounds (see details in the supporting information), we first performed absorption spectra and fluorescent studies (Fig. 2). Probes 2 and 4 exhibited similar absorption spectra with a maximum absorption at 340 nm (Fig. 2A and 2C). Compounds 1 and 3 showed maximum absorption at 390 nm and 380 nm, respectively. The blue shift of absorption of azide analogs may be caused by the stronger electron withdrawing property of azido group. In the fluorescent spectra studies, as shown in Fig. 2B and 2D, compounds 1 and 3 produced maximum emission at 500 nm and 550 nm, respectively, when excited at 405 nm. Notably, no fluorescence emission was observed for both probes 2 and 4. While compound 3 showed a larger stoke shift, compound 1 exhibited much stronger fluorescence with quantum yield 30-fold higher than compound 3 at excitation of 405 nm (Fig. S1 and Table S1). There is little change of the fluorescence over pH 4.5–8.5, while at pH 9.5 and 10.5 the fluorescence intensity decreased significantly (Fig. S2). The stability of the probe may be not good at basic conditions, however, they still stable at a wide physiological pH range. Therefore, probe 2 was selected for further studies.

To confirm the chemical reactivity of 2 towards H₂S, we incubated NaHS (100 μM) with probe 2 (10 μM) for different time periods at 37 °C in PBS at pH 7.4 to mimic physiological conditions. As shown in Fig. 3A, significant fluorescence can be detected within 20 min, and the fluorescence intensities steadily increased with incubation time, which clearly indicates the potential of probe 2 in detecting H₂S under physiological conditions. Next, we performed fluorescence titration studies of probe 2 under the same conditions with series of concentrations of NaHS (0.25–100 μM) for 60 min (excitation 405 nm and emission 500

nm). As shown in Fig. 3B, the free probe 2 is non-fluorescent under the testing conditions. Notably, the fluorescence intensities steadily increased with the addition of increasing concentrations of NaHS. More importantly, the fluorescence intensities are linearly correlated with the concentration of HS^- . The detection limit, based on a signal-to-background ratio of 3, was estimated to be 3.1 μM .

For practical application in complex biological systems, the selectivity of the probe is essential. Therefore, we next evaluated the specific nature of this probe for H_2S . To this end, various species, including reductive sulfur species (Na_2SO_3 , $\text{Na}_2\text{S}_2\text{O}_3$), reductive sulfur species in biological systems (Cys, GSH), reactive oxygen species (H_2O_2), reactive nitrogen species (NaNO_2), and some commonly encountered ions in biological systems (NaCl, NaF, NaN_3 , NaOAc), were chosen for this study and compared with NaHS. As shown in Fig. 4, the common reactive sulfur, nitrogen, and oxygen species did not induce fluorescence upon incubation with probe 2. Notably, the biologically relevant sulfur species, such as GSH and Cys, did not reduce probe 2 and elicit fluorescent emission either, thus suggesting that they will not interfere with the detection of HS^- in biological systems by probe 2. While SO_3^{2-} enhanced fluorescence upon incubation with probe 2, the concentration needed for SO_3^{2-} (1 mM) is significantly higher and the induced-fluorescence intensity is significantly lower compared to HS^- . Collectively, these results strongly suggest high selectivity of probe 2.

With these promising results in hand, we moved on to investigate whether probe 2 has the potential to detect H_2S in biological samples. To accomplish this, we first evaluated the chemical transformation of probe 2 by NaHS (0.25–100 μM) in fetal bovine serum (FBS). Surprisingly, fluorescent turn-on can only be detected when the NaHS concentration is higher than 20 μM , and these results fit a polynomial model (Fig. S3). This could be due to the quenching of H_2S by FBS or proteins binding upon production of 1, as similar effects have been reported^{31–33}. To confirm this, we incubated compound 1 at a series of concentrations with FBS. The results demonstrated that FBS can partially quench the fluorescence of 1 (Fig. S4). Considering the potential applications of probe 2 in cell-based models, we further studied the induction of fluorescence of probe 2 (10, 50, and 100 μM) by NaHS (0.25–100 μM) in cellular growth medium (10% FBS in RPMI1640) at 37 °C for 60 min. As shown in Fig. 5, a linear relationship with NaHS concentration was established under these experimental settings, thus suggesting that probe 2 could be applied to detect H_2S in living cells.

Lastly, we investigated the application of probe 2 in U266 cells. As shown in Fig. 6A, flow cytometry analysis demonstrated that U266 cells, pretreated with probe 2 (50 μM) for 1 h, gave strong fluorescence after exposure to NaHS (100 μM), compared to probe 2 alone. Microscopic analysis using an imaging flow cytometer also demonstrated the induction of green fluorescence inside of the cells upon addition of NaHS (Fig. 6B). The cytotoxicity of Pomal- N_3 was evaluated by MTT assay (Fig. S7). After incubation with U266 cells for 24h, Pomal- N_3 showed no significant cytotoxicity on U266 cells at 10, 50, and 100 μM . Taken together, these results strongly suggest that probe 2 can penetrate the cell membrane and is safe to be used to detect H_2S in living cells.

In summary, we have developed an azide analog of pomalidomide (probe 2) as a fluorescent turn-on probe for H₂S based on the azide reduction principle. This probe can be prepared easily through three steps with high yield and can be modified easily. Our studies demonstrated high selectivity and sensitivity of this probe towards H₂S. Furthermore, flow cytometry and imaging studies in U266 cells indicated that probe 2 can be successfully applied in living cells to detect H₂S. Because of the easy access and the flexibility of structural modifications of the 3-aminophthalimide fluorophore, this probe represents a promising starting point for further development of organelle specific H₂S probes, which will greatly facilitate the understanding of pathophysiological roles of H₂S.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Chemical structure of parent fluorescent compounds and probes

Figure 2.

Spectra of probes at 10 μM in PBS at pH 7.4. (A) UV absorption of 4-aminothalidomide and 4-azidothalidomide. (B) Emission spectra of 4-aminothalidomide and 4-azidothalidomide at an excitation of 380 nm. (C) UV absorption of pomalidomide and Pomal-N₃. (D) Emission spectra of pomalidomide and Pomal-N₃ at an excitation of 405 nm.

Figure 3.

(A) Time dependent fluorescence intensities increase of Pomal-N₃ (10 μM) at an emission of 500 nm (excitation: 405 nm) after addition of 100 μM NaHS in PBS at pH7.4. (B) Linear relationship between Pomal-N₃ (10 μM) fluorescence at 500 nm and NaHS (0–100 μM) in PBS at pH7.4.

Figure 4.

Pomal-N₃ (10 μ M) was incubated with NaHS (100 μ M), Na₂SO₃ (100 μ M and 1 mM), and NaOAc, NaCl, NaF, NaN₃, Na₂S₂O₃, Na₂SO₄, GSH, Cys, NaNO₂, H₂O₂ (each at 1 mM) in PBS at pH7.4 for 60 min. (A) Fluorescence spectra. (B) Fluorescence intensities at 500 nm.

Figure 5. Pomal-N₃ (10, 50, and 100 μM) was incubated with NaHS (0–100 μM) in U266 cellular growth medium (10% FBS in RPMI1640).

Figure 6.

U266 cells were preincubated with 50 μ M Pomal-N₃ for 1 h, then further incubated with NaHS (100 μ M) for 30 min. (A) The samples were analyzed by flow cytometry with blue laser (405 nm) and green filter (530 nm). (B) Green fluorescence images (ex: 405 nm) of cells treated with Pomal-N₃ only (columns a, c) and corresponding bright field images (columns b, d); Green fluorescence images (ex: 405 nm) of cells treated with Pomal-N₃ followed by NaHS (columns e, g) and corresponding bright field images (columns f, h).