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Hydrogen Sulfide (H₂S) Donors Activated by Reactive Oxygen Species (ROS)

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Abstract

Hydrogen sulfide (H₂S) exhibits promising protective effects in many (patho)physiological processes, as evidenced by recent reports using synthetic H₂S donors in different biological models. Herein, we report the design and evaluation of **PeroxyTCM**, which comprises the first class of reactive oxygen species (ROS)-triggered H₂S donors. These donors are stable in aqueous solution and do not release H₂S until triggered by ROS, such as hydrogen peroxide (H₂O₂), superoxide (O₂^{•−}), and peroxynitrite (ONOO[−]). We demonstrate ROS-triggered H₂S donation in live cells and also demonstrate that **PeroxyTCM-1** provides protection against H₂O₂-induced oxidative damage, suggesting potential future applications of **PeroxyTCM** and similar scaffolds in H₂S-related therapies.

Graphical Abstract

H₂S is an important biomolecule and H₂S donors are valuable research and pharmacological tools. We report the design and cellular evaluation of an H₂S donor that is triggered to release H₂S by exposure to reactive oxygen species (ROS).

Keywords

hydrogen sulfide; thiocarbamate; carbonyl sulfide; reactive oxygen species; oxidative stress

Hydrogen sulfide (H₂S) is now recognized as an important cellular signaling molecule due to its important functions in various aspects of human health and disease, and also as a member of the gasotransmitter family along with nitric oxide (NO) and carbon monoxide (CO).^[1] Biological H₂S is generated primarily from Cys and/or Hcy by cystathionine γ -lyase (CSE), cystathionine β -synthase (CBS), cysteine aminotransferase (CAT), and 3-mercaptopyruvate sulfur transferase (3-MST), which work either individually or in concert.^[2] In many cases, both endogenous H₂S production, as well as exogenous H₂S

administration, has been demonstrated to protect cells, tissues, and organs against damage associated with different (patho)physiological processes.^[3] For example, H₂S shows potent anti-inflammation effects in animal models^[5] and exhibits antioxidant properties and protective effects against reactive oxygen species (ROS).^[6] Additionally, H₂S provides protection against myocardial ischemia reperfusion (MI/R) injury by consuming ROS generated by dysfunctional mitochondria and thus preserving cardiac activity.^[7]

Many researchers use H₂S-releasing small molecules (“H₂S donors”) as primary tools to modulate cellular H₂S levels (Figure 1).^[8] Although exogenous administration of Na₂S or NaHS provides a convenient source of H₂S, the instantaneous and uncontrollable H₂S release from these salts does not mimic endogenous generation and often result in acute side effects and contradictory results (i.e. pro- and anti-inflammatory effects).^[9] Two of the most commonly used H₂S donor classes include polysulfides derived from natural products, such as diallyl trisulfide (DATS),^[10] and hydrolysis-based donors, such as GYY4137,^[11] derived from Lawesson’s reagent, both of which exhibit H₂S-related protective effects in a wide array of systems.^[8b–c] Additionally, dithiolethione (ADT) and its derivative ADT-OH have been used to develop a series of H₂S-hybrid nonsteroidal anti-inflammation drugs, which greatly reduce GI damage while maintaining NSAID activity.^[12] Synthetic thiol-activated H₂S donors have also been developed based on protected disulfides, with some exhibiting promising protective effects in animal models.^[13] More recently, donors based on esterase-activation^[14] and pH-modulation^[15] have been reported, and demonstrated to influence inflammatory response factors and provide protection in oxidative stress models, respectively. In addition, other H₂S-donating motifs, such as thioamino acids,^[16] caged *gem*-dithiols,^[17] and caged ketoprofenate,^[18] are also being investigated for different applications. Despite the diverse palette of available donor motifs, two main challenges remain. First, many synthetic donors lack appropriate, H₂S-depleted control compounds, which complicates conclusions drawn from use of these donors. Second, few donors can be triggered by specific cellular species or events, thus limiting the tunability of available platforms. Based on these needs, H₂S donors that respond to specific stimuli and have suitable control compounds would provide a significant advance.

A viable platform to access such responsive H₂S donors stems from related H₂S sensing work recently report from our lab, in which a new class of analyte-replacement fluorescent probes was developed using the engineered release of carbonyl sulfide (COS) from thiocarbamates.^[19] Importantly, probe activation generated a fluorescence response and also released COS, which is quickly hydrolyzed to H₂S by carbonic anhydrase (CA), a ubiquitous enzyme in plants and mammals. Recognizing the potential utility that similar platforms could provide for triggered H₂S release, we envisioned that caged-thiocarbamates could also serve as a new and diverse class of H₂S donors that could be engineered to release H₂S in response to specific stimuli. Importantly, these donors would operate by mechanisms dissimilar to currently-available H₂S donors, and would enable access to viable H₂S-depleted control compounds absent from most donor constructs, thus addressing major limitations in the field. Here we report the use of caged thiocarbamates, in combination with ROS-responsive arylboronate triggers, to access the first class of triggerable H₂S donors activated by cellular ROS (Scheme 1a).

To test our hypothesis that thiocarbamate functionalized arylboronates could function as ROS-triggered H₂S donors, we prepared three thiocarbamate donors (PeroxyThioCarbaMate: **PeroxyTCM-1**, **PeroxyTCM-2**, and **PeroxyTCM-3**) and two carbamate control compounds (ThioCarbaMates: **TCM-1** and **TCM-2**). The **PeroxyTCM** compounds are stable in aqueous buffer (pH 5 – 9) and are not hydrolyzed by esterases. We also prepared the parent carbamate (PeroxyCarbaMate-1, **PeroxyCM-1**), which can also be activated by ROS, but releases CO₂/H₂O instead of COS/H₂S. Access to these simple control compounds provides useful tools to determine whether observed biological activities of the donors are H₂S-related or merely a product of the organic scaffold and/or byproducts.

To evaluate the H₂S release from the donor constructs in the presence of ROS, we used an H₂S-selective electrode to monitor H₂S release from **PeroxyTCM-1** (50 μM) upon treatment with H₂O₂ (50 – 1000 μM) in PBS buffer (pH 7.4, 10 mM) containing CA (25 μg/mL). Consistent with our hypothesis, we observed H₂O₂-dependent H₂S release from **PeroxyTCM-1** with corresponding second order rate constant of 1.44 M⁻¹s⁻¹ (Figure 2a, S1). Quantification of H₂S release 50 μM **PeroxyTCM-1** using electrode data demonstrated a H₂S release efficiencies of 80% and 60% in the presence of 250 μM and 500 μM H₂O₂, respectively, which are consistent with increased H₂O₂ scavenging by H₂S at higher ROS concentrations. We next evaluated **PeroxyTCM-2** and **PeroxyTCM-3** and demonstrated that the rate of H₂S release can be tuned by electronic modulation of the thiocarbamate (Figure 2b). By contrast, **TCM-1** and **TCM-2**, which lack the H₂O₂-reactive arylboronate trigger, failed to release H₂S upon treatment with H₂O₂ (Figure 2b). Taken together, these studies demonstrate that arylboronate-functionalized thiocarbamates provide a functional platform to access H₂O₂-mediated H₂S donors.

We investigated whether CA was essential to convert COS to H₂S by incubating **PeroxyTCM-1** with H₂O₂ (10 equiv.) in the absence of CA. Although COS can be hydrolyzed to H₂S under both acidic and basic conditions, this hydrolysis is much slower at physiological pH.^[20] Unexpectedly, a positive H₂S release response was observed, indicating that COS could react directly with H₂O₂ to generate H₂S in a CA-independent pathway (Figure S2a, red and blue lines). To further investigate these observations, we treated an aqueous solution (10 mM PBS, pH 7.4) of COS gas with H₂O₂. No H₂S was observed prior to H₂O₂ addition, whereas H₂O₂ addition resulted in rapid H₂S generation (Figure S2b). Notably, these studies demonstrate that H₂O₂ alone can convert COS to H₂S directly, although this process was significantly slower than CA-catalyzed COS hydrolysis.

We next evaluated which specific reactive sulfur, oxygen, and nitrogen species (RSONS) resulted in donor activation by measuring H₂S release from **PeroxyTCM-1** after incubation with different RSONS (Figure 3). We found that incubation with H₂O₂, O₂⁻, or ONOO⁻ resulted in H₂S release, with H₂O₂ being the most active trigger. Other RSONS, such as hypochlorite (ClO⁻), hydroxyl radical (HO·), singlet oxygen (¹O₂), tert-butyl hydroperoxide (TBHP), tert-butoxy radical (tBuO·) cysteine (Cys), reduced glutathione (GSH), oxidized glutathione (GSSG), S-nitrosoglutathione (GSNO), nitrite (NO₂⁻), sulfate (SO₄²⁻), thiosulfate (S₂O₃²⁻), NO, or nitroxyl (HNO) failed to release H₂S.^[21] Taken together, this selectivity screening demonstrates that only specific ROS (H₂O₂, O₂⁻, and ONOO⁻) activate **PeroxyTCM-1** to release H₂S.

Before investigating different potential biological applications of the **PeroxyTCM** compounds, we first investigated the cytotoxicity of **PeroxyTCM-1**, **PeroxyCM-1** and **TCM-1** (10 – 100 μM) in HeLa cells. No significant decrease in cell viability was observed after a 2 hour incubation, indicating that none of the three compounds exhibited appreciable cytotoxicity at the tested concentrations (Figure S3). We next investigated whether exogenous H_2O_2 could be used to release H_2S in cellular environments by incubating HeLa cells with **PeroxyTCM-1** (50 μM) followed by treatment with H_2O_2 (25 μM or 50 μM). We used **HSN2**, a reaction-based H_2S fluorescent probe, to monitor H_2S accumulation by fluorescence microscopy.^[22] In the absence of H_2O_2 , no **HSN2** fluorescence was observed, confirming that **PeroxyTCM-1** was stable and did not release H_2S in a normal cellular environment. By contrast, addition of H_2O_2 resulted in a H_2O_2 dose-dependent increase in **HSN2** fluorescence (Figures 4 and S5), confirming that **PeroxyTCM-1** can be activated by exogenous ROS in a cellular environment to release H_2S .

Having demonstrated activation by exogenous ROS, we next investigated the response of **PeroxyTCM-1** to endogenous ROS generation. RAW 264.7 cells were incubated with phorbol 12-myristate 13-acetate (PMA), which is a well-established method to induce ROS and H_2O_2 production in macrophages.^[23] ROS generation was confirmed using 2',7'-dichlorofluorescein diacetate (DCFDA) (Figure S6). **PeroxyTCM-1**-treated cells were stimulated by PMA, and H_2S release was monitored using **HSN2**. In the absence of PMA, no fluorescent signal from **HSN2** was observed. By contrast, addition of 500 nM PMA resulted in a significant increase in signal from **HSN2** corresponding to the released H_2S (Figure 5). These studies confirm that **PeroxyTCM-1** is sensitive enough to be activated by endogenous ROS, suggesting that it may provide a viable platform for ROS-related H_2S investigations.

In addition to cellular imaging experiments, we also investigated whether the developed ROS-activated donors could provide protection against ROS-related oxidative stress in simple cell culture models. Recent studies suggest that ROS play deleterious roles in various physiological and pathological systems ranging from aging to cardiovascular damage. In many cases, H_2S administration can provide partial protection or rescue from these different disease states. For example, ROS generated during mitochondrial dysfunction are responsible for a wide range of damages in the cardiovascular system, including MI/R injury, and that exogenous H_2S significantly preserved cardiac activity through a ROS scavenging pathways.^[7b, 24] On the basis of this H_2S / ROS relationship, we envisioned that **PeroxyTCM** compounds would exhibit similar cytoprotective effects toward ROS-induced damage due to H_2S release.

To simulate increased cellular oxidative stress, we incubated HeLa cells with H_2O_2 (50 – 400 μM) for 1 hour and observed a dose-dependent reduction of cell viability (Figure 6a). Since 100 μM of H_2O_2 led to ~70% of cell death, this dose was used to investigate protective activities of **PeroxyTCMs**. Although this dose of H_2O_2 does not represent physiological levels of H_2O_2 , it falls into the range of H_2O_2 concentrations used to induce oxidative stress in previous studies. In subsequent experiments, cells were treated with H_2O_2 (100 μM) in the presence or absence of **PeroxyTCM-1**, **PeroxyCM-1**, or **TCM-1** (10 – 50 μM) for 1 hour. As expected, **PeroxyTCM-1** exhibited a significant dose-dependent increase of cell

viability, suggesting that the released H₂S provided rescue from H₂O₂-induced oxidative damage (Figure 6b). **PeroxyCM-1** showed an attenuated rescue from oxidative stress (Figure 6c) due to H₂O₂ consumption by the arylboronate and antioxidant effects of 4-hydroxylbenzyl alcohol (HBA), one of the byproducts after H₂S generation (Figure S3),^[25] and the observed protection was significantly lower than that from **PeroxyTCM-1** (Figure S7). By contrast, **TCM-1** provided no protection against H₂O₂-mediated oxidative stress (Figure 6d). Taken together, these results provide strong evidence that **PeroxyTCM-1** is a robust H₂S donor and provides cellular protection from oxidative stress. In addition, compared to other H₂S donors, specific ROS selectivity makes the targeting of ROS-triggered H₂S donors to different subcellular locations feasible, which would greatly benefit the H₂S-related biological investigations and H₂S-based therapeutics development.

In summary, we provide the first example of ROS-triggered H₂S donors. These donors operate by mechanisms orthogonal to available H₂S donors and provide access to suitable control compounds for biological studies. Initial proof-of-concept studies reveal that **PeroxyTCM-1** exhibits promising cytoprotective activities against H₂O₂-induced oxidative stress, suggesting future potential applications of these and similar constructs as prodrugs in H₂S-related therapies. Further applications of the present as well as related COS-related H₂S donors triggered by other mechanisms are currently ongoing in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Selected H₂S donating molecules and motifs.

Figure 2.

(a) H₂S release from **PeroxyTCM-1** (50 μ M) in the presence of H₂O₂ (50 – 1000 μ M) in PBS (pH 7.4, 10 mM) containing CA (25 μ g/mL). (b) H₂S release from thiocarbamates (50 μ M) in the presence of H₂O₂ (500 μ M) in PBS (pH 7.4, 10 mM) containing CA (25 μ g/mL).

Figure 3.

H₂S release response of **PeroxyTCM-1** (50 μ M) to various RSONS (5 mM for GSH, 500 μ M for all other RSONS) in PBS buffer (pH 7.4, 10 mM; 100 mM for GSH and ONOO⁻). Experiments were performed at r.t. for 20 min. The response is expressed as mean $\pm$ SD (n = 3).

Figure 4.

H_2S release from **PeroxyTCM-1** in HeLa cells. HeLa cells were co-incubated with **PeroxyTCM-1** (50 μM), **HSN2** (5 μM), and NucBlue nuclear dye for 30 min. After removal of extracellular **PeroxyTCM-1** and **HSN2**, cells were incubated in FBS-free DMEM in the absence (Top row) or presence (Bottom row) of H_2O_2 (50 μM) for 30 min.

Figure 5.

H₂S release from **PeroxyTCM-1** in RAW 264.7 cells. Cells were co-incubated with **PeroxyTCM-1** (50 μM), **HSN2** (5 μM) and NucBlue dye for 30 min. After washing, cells were incubated in FBS-free DMEM in the absence (Top row) or presence (Bottom row) of PMA (500 nM) for 3 h.

Figure 6.

(A) Cytotoxicity of H₂O₂ (50 – 400 μM) in HeLa cells. Cytoprotections of **PeroxyTCM-1** (B), **PeroxyCM-1** (C), and **TCM-1** (D) against H₂O₂-induced (100 μM) oxidative stress in HeLa cells. Results were expressed by mean ± SEM (n = 5). *****P* < 0.0001 vs VEH group; ##*P* < 0.01, ###*P* < 0.001, and ####*P* < 0.0001 vs H₂O₂-treated group, respectively.

Scheme 1. Design (a) and synthesis (b) of ROS-triggered H_2S donors.