H_2S Donors

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Hydrogen Sulfide Donors Activated by Reactive Oxygen Species

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Abstract: *Hydrogen sulfide (H_2S) exhibits promising protective effects in many (patho)physiological processes, as evidenced by recent reports using synthetic H_2S donors in different biological models. Herein, we report the design and evaluation of compounds denoted PeroxyTCM, which are the first class of reactive oxygen species (ROS)-triggered H_2S donors. These donors are engineered to release carbonyl sulfide (COS) upon activation, which is quickly hydrolyzed to H_2S by the ubiquitous enzyme carbonic anhydrase (CA). The donors are stable in aqueous solution and do not release H_2S until triggered by ROS, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and peroxynitrite ($ONOO^-$). We demonstrate ROS-triggered H_2S donation in live cells and also demonstrate that PeroxyTCM-1 provides protection against H_2O_2-induced oxidative damage, suggesting potential future applications of PeroxyTCM and similar scaffolds in H_2S-related therapies.*

Hydrogen sulfide (H_2S) is now recognized as an important cellular signaling molecule owing 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 H_2S 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 H_2S production, as well as exogenous H_2S administration, has been demonstrated to protect cells, tissues, and organs against damage associated with different (patho)physiological processes.[3,4] For example, H_2S shows potent *anti*-inflammation effects in animal models[5] and exhibits antioxidant properties and protective effects against reactive oxygen species (ROS).[6] Additionally, H_2S 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 H_2S-releasing small molecules ("H_2S donors") as primary tools to modulate cellular H_2S levels (Figure 1).[8] Although exogenous administration of Na_2S or NaHS provides a convenient source of H_2S, the instantaneous and uncontrollable H_2S 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 H_2S 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 H_2S-related protective effects in a wide array of systems.[8b–e] Additionally, dithiolethione (ADT) and its derivative ADT-OH have been used to develop a series of H_2S-hybrid nonsteroidal *anti*-inflammation drugs, which greatly reduce GI damage while maintaining NSAID activity.[12] Synthetic thiol-activated H_2S 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 H_2S-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, H_2S-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, H_2S donors that respond to specific stimuli and have suitable control compounds would provide a significant advance.

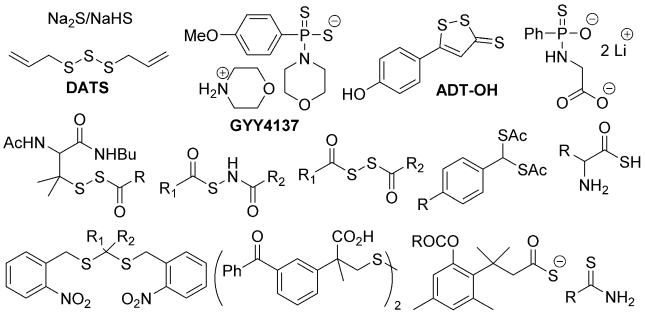

Figure 1. Selected H_2S donating molecules and motifs.

A viable platform to access such responsive H_2S donors stems from related H_2S sensing work recently report by us, 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

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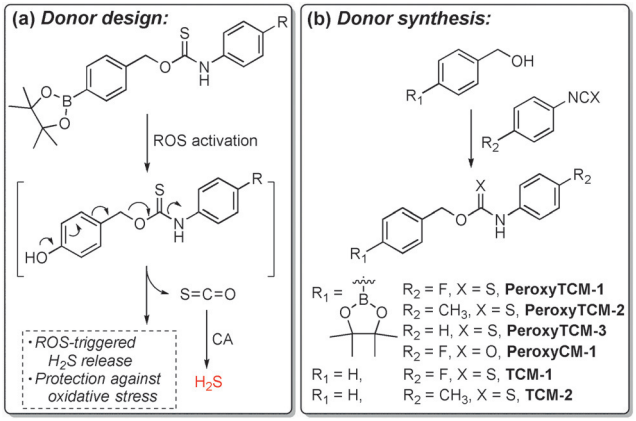
released COS, which is quickly hydrolyzed to H_2S by carbonic anhydrase (CA), a ubiquitous enzyme in plants and mammals. Recognizing the potential utility that similar platforms could provide for triggered COS/H_2S release, we envisioned that caged-thiocarbamates could also serve as a new and diverse class of H_2S donors that could be engineered to release H_2S in response to specific stimuli. Importantly, these donors would operate by mechanisms dissimilar to currently available H_2S donors, and would enable access to viable H_2S-depleted control compounds absent from most donor constructs, thus addressing major limitations in the field. Herein we report the use of caged thiocarbamates, in combination with ROS-responsive arylboronate triggers, to access the first class of triggerable COS/H_2S donors activated by cellular ROS (Scheme 1 a).



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

To test our hypothesis that thiocarbamate functionalized arylboronates could function as ROS-triggered H_2S 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 CO_2/H_2O instead of COS/H_2S. Access to these simple control compounds provides useful tools to determine whether observed biological activities of the donors are H_2S-related or merely a product of the organic scaffold and/or byproducts.

To evaluate the H_2S release from the donor constructs in the presence of ROS, we used an H_2S-selective electrode to monitor H_2S release from PeroxyTCM-1 (50 μM) upon treatment with H_2O_2 (50–1000 μM) in PBS buffer (pH 7.4, 10 mM) containing CA (25 μg mL^{-1}). Consistent with our hypothesis, we observed H_2O_2-dependent H_2S release from PeroxyTCM-1 with corresponding second-order rate constant of 1.44 M^{-1} s^{-1} (Figure 2 a, Figure S1 in the Supporting Information). Quantification of H_2S release, 50 μM PeroxyTCM-1 using electrode data demonstrated a H_2S release efficiencies of 80 % and 60 % in the presence of 250 μM and 500 μM H_2O_2,

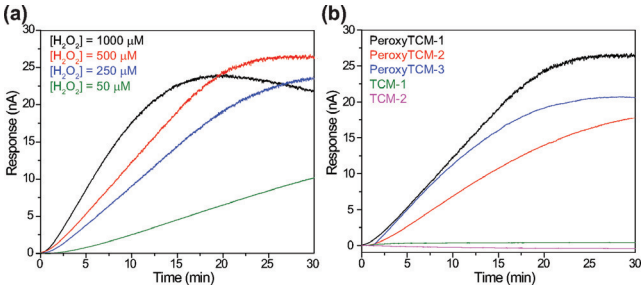


Figure 2. a) H_2S release from PeroxyTCM-1 (50 μM) in the presence of H_2O_2 (50–1000 μM) in PBS (pH 7.4, 10 mM) containing CA (25 μg mL^{-1}). b) H_2S release from thiocarbamates (50 μM) in the presence of H_2O_2 (500 μM) in PBS (pH 7.4, 10 mM) containing CA (25 μg mL^{-1}).

respectively, which are consistent with increased H_2O_2 scavenging by H_2S at higher ROS concentrations. We next evaluated PeroxyTCM-2 and PeroxyTCM-3 and demonstrated that the rate of H_2S release can be tuned by electronic modulation of the thiocarbamate (Figure 2 b). In contrast, TCM-1 and TCM-2, which lack the H_2O_2-reactive arylboronate trigger, failed to release H_2S upon treatment with H_2O_2 (Figure 2 b). Taken together, these studies demonstrate that arylboronate-functionalized thiocarbamates provide a functional platform to access H_2O_2-mediated H_2S donors.

We investigated whether CA was essential to convert COS into H_2S by incubating PeroxyTCM-1 with H_2O_2 (10 equiv) in the absence of CA. Although COS can be hydrolyzed to H_2S under both acidic and basic conditions, this hydrolysis is much slower at physiological pH.[20] Unexpectedly, a positive H_2S release response was observed, indicating that COS could react directly with H_2O_2 to generate H_2S in a CA-independent pathway (Figure S2a). To further investigate these observations, we treated an aqueous solution (10 mM PBS, pH 7.4) of COS gas with H_2O_2. No H_2S was detected prior to H_2O_2 addition, whereas H_2O_2 addition resulted in rapid H_2S generation (Figure S2b). Notably, these studies demonstrate that H_2O_2 alone can convert COS into H_2S 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 H_2S release from PeroxyTCM-1 after incubation with different RSONS (Figure 3). We found that incubation with H_2O_2, O_2^-, or $ONOO^-$ resulted in H_2S release, with H_2O_2 being the most active trigger. Other RSONS, such as hypochlorite (ClO^-), hydroxyl radical (HO·), singlet oxygen ($^1O_2$), *tert*-butyl hydroperoxide (TBHP), *tert*-butoxy radical (tBuO·) cysteine (Cys), reduced glutathione (GSH), oxidized glutathione (GSSG), S-nitrosoglutathione (GSNO), nitrite (NO_2^-), sulfate (SO_4^{2-}), thiosulfate ($S_2O_3^{2-}$), NO, or nitroxyl (HNO) failed to release H_2S.[21] Taken together, this selectivity screening demonstrates that only specific ROS (H_2O_2, O_2^-, and $ONOO^-$) activate PeroxyTCM-1 to release H_2S.

Before investigating different potential biological applications of the PeroxyTCM compounds, we first investigated to cytotoxicity of PeroxyTCM-1, PeroxyCM-1 and TCM-

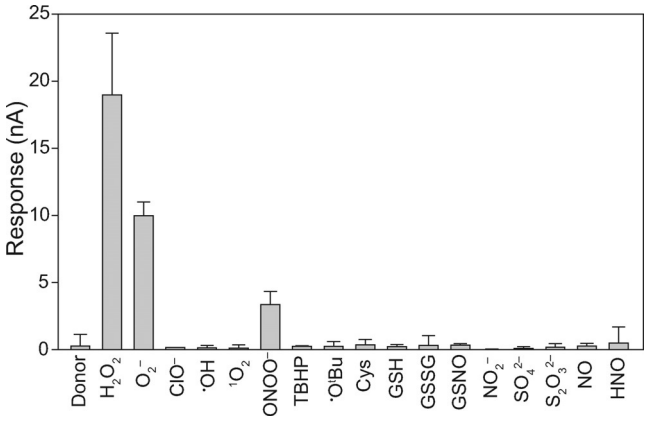


Figure 3. H_2S release response of PeroxyTCM-1 (50 μM) to various RSONS (5 mM for GSH, 500 μM for all other RSONS) in PBS buffer (pH 7.4, 10 mM; 100 mM for GHS and $ONOO^-$). Experiments were performed at room temperature for 20 min. The response is expressed as mean ± SD ($n = 3$).

1 (10–100 μM) in HeLa cells. No significant decrease in cell viability was observed after a 2 h 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 Figure S5), confirming that PeroxyTCM-1 can be activated by exogenous ROS in a cellular environment to release H_2S.

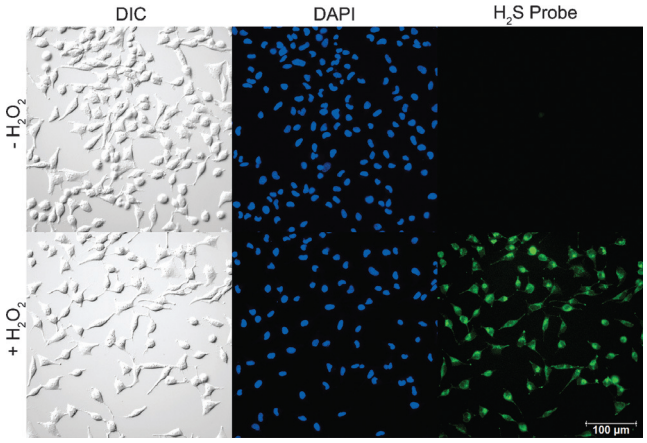


Figure 4. H_2S release from PeroxyTCM-1 in HeLa cells. HeLa cells were co-incubated with PeroxyTCM-1 (50 μM), HSN2 (5 μM), and NucBlue nuclear dye for 30 min. After removal of extracellular PeroxyTCM-1 and HSN2, cells were incubated in FBS-free DMEM in the absence (Top row) or presence (Bottom row) of H_2O_2 (50 μM) for 30 min.

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′-dichlorofluorescin 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.

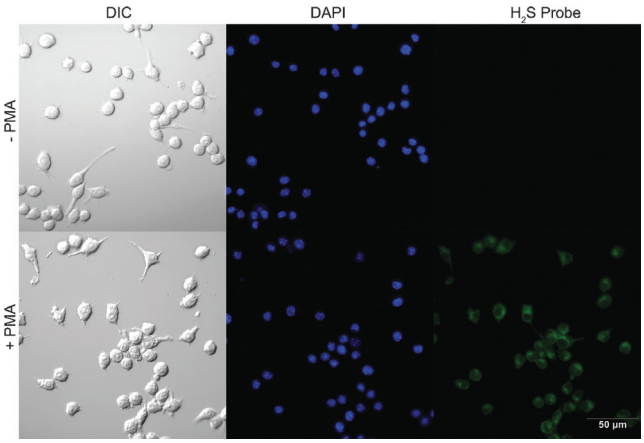


Figure 5. H_2S release from PeroxyTCM-1 in RAW 264.7 cells. Cells were co-incubated with PeroxyTCM-1 (50 μM), HSN2 (5 μM), and NucBlue dye for 30 min. After washing, cells were incubated in FBS-free DMEM in the absence (Top row) or presence (Bottom row) of PMA (500 nM) for 3 h.

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 damage 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 h and observed a dose-dependent reduction of cell viability (Figure 6a). Since 100 μM of H_2O_2 led to approximately 70% cell death, this

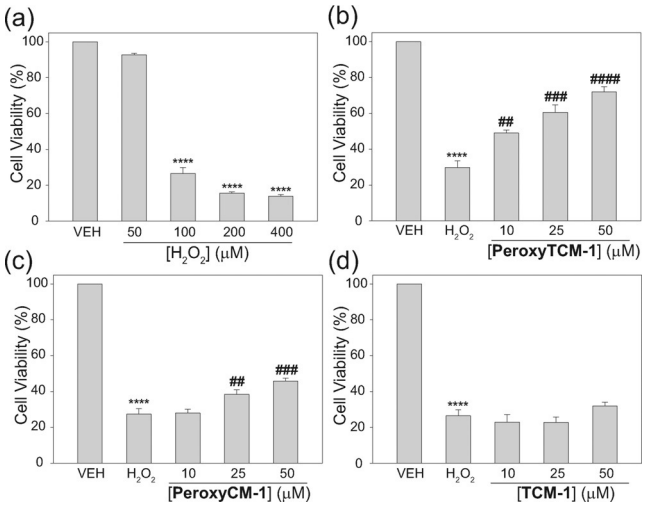


Figure 6. a) Cytotoxicity of H_2O_2 (50–400 μM) in HeLa cells. Cytoprotections of b) PeroxyTCM-1, c) PeroxyCM-1, and d) TCM-1 against H_2O_2-induced (100 μM) oxidative stress in HeLa cells. Results were expressed by mean ± SEM ($n=5$). $^{****}P<0.0001$ vs. VEH group; $^{##}P<0.01$, $^{###}P<0.001$, and $^{####}P<0.0001$ vs. H_2O_2-treated group, respectively.

dose was used to investigate protective activities of PeroxyTCMs. Although this dose of H_2O_2 is higher than physiological H_2O_2 concentrations, 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 h. As expected, PeroxyTCM-1 exhibited a significant dose-dependent increase of cell viability, suggesting that the released H_2S provided rescue from H_2O_2-induced oxidative damage (Figure 6b). PeroxyCM-1 showed an attenuated rescue from oxidative stress (Figure 6c) as a result of H_2O_2 consumption by the arylboronate and antioxidant effects of 4-hydroxylbenzyl alcohol (HBA), one of the byproducts after H_2S generation (Figure S3),[25] but the observed protection was significantly lower than that from PeroxyTCM-1 (Figure S7). In contrast, TCM-1 provided no protection against H_2O_2-mediated oxidative stress (Figure 6d). Taken together, these results provide strong evidence that PeroxyTCM-1 is a robust H_2S donor and provides cellular protection from oxidative stress. In addition, compared to other H_2S donors, specific ROS selectivity makes the targeting of ROS-triggered H_2S donors to different subcellular locations feasible, which would greatly benefit the H_2S-related biological investigations and H_2S-based therapeutics development.

In summary, we provide the first example of ROS-triggered H_2S donors. These donors operate by mechanisms orthogonal to available H_2S 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 H_2O_2-induced oxidative stress, suggesting future potential applications of these and similar constructs as prodrugs in H_2S-related therapies. Further applications of the present as well as related COS-related H_2S donors triggered by other mechanisms are ongoing.

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