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Esterase-Triggered Self-Immulative Thiocarbamates Provide Insights into COS Cytotoxicity

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Abstract

Hydrogen sulfide (H_2S) is an important gasotransmitter and biomolecule, and many synthetic small-molecule H_2S donors have been developed for H_2S -related research. One important class of triggerable H_2S donors are self-immolative thiocarbamates, which function by releasing carbonyl sulfide (COS), which is rapidly converted to H_2S by the ubiquitous enzyme carbonic anhydrase (CA). Prior studies of esterase-triggered thiocarbamate donors reported significant inhibition of mitochondrial bioenergetics and toxicity when compared to direct sulfide donors, suggesting that COS may function differently than H_2S . Here, we report a suite of modular esterase-triggered self-immolative COS donors and include the synthesis, H_2S release profiles, and cytotoxicity of the developed donors. We demonstrate that the rate of ester hydrolysis correlates directly with the observed cytotoxicity in cell culture, which further supports the hypothesis that COS functions as more than a simple H_2S shuttle in certain biological systems.

Graphical Abstract

INTRODUCTION

Hydrogen sulfide (H_2S), the most recent addition to the gasotransmitter family,¹ plays important physiological roles in the cardiovascular,² respiratory, as well as other organ systems.³ Significant interest in both research and therapeutic approaches for H_2S delivery has led to the development of a library of synthetic small molecules that release H_2S (H_2S donors) by using different strategies.^{4–8} In one recently-developed approach, our group, as well as others, has reported H_2S donors based on the triggerable, self-immolative decomposition of thiocarbamates to release carbonyl sulfide (COS), which is rapidly

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hydrolyzed to H₂S by the ubiquitous mammalian enzyme carbonic anhydrase (CA).⁹ This COS-dependent H₂S-releasing strategy is highly tunable and allows for triggering of H₂S release by a variety of stimuli, including ROS,^{10–11} nucleophiles,¹² cysteine,¹³ and light.^{14–16}

In addition to functioning as a precursor for CA-mediated H₂S release, COS is the most prevalent sulfur-containing gas in Earth's atmosphere and plays important roles in the global sulfur cycle. Despite this significance, few studies have investigated the physiological properties of COS directly.¹⁷ Currently, there are no established mechanisms of eukaryotic COS biosynthesis, although it has been shown that acetylcholine stimulation of porcine coronary artery (PCA) leads to an observed increase in COS, indicating that muscarinic acetylcholine receptors could play a role in regulating COS synthesis.¹⁸ Additionally, it has been detected in the headspace of PCA and cardiac muscle,¹⁸ suggesting potential endogenous production. Although simple methods for the direct detection of COS in aqueous solutions are not currently available, COS can be detected through GC-MS analysis or by other spectroscopic methods. Moreover, COS has also been recognized as a potential exhaled breath biomarker for a variety of diseases, including cystic fibrosis¹⁹ as well as liver disease and rejection,^{20–21} which suggests a possible role in disease physiology. The consumption of COS by CA is well established and COS toxicity closely resembles that of H₂S, which is likely due to CA-mediated hydrolysis within mucous membranes upon exposure. The rapid conversion of COS to H₂S, with an associated rate constant of $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (for bovine CA II), makes COS a convenient source of sulfide, but also makes disentangling the chemical biology of COS from H₂S inherently challenging.²²

We recently reported an esterase-triggered COS-mediated H₂S donor,²³ wherein ester cleavage reveals an intermediate phenol that undergoes a 1,4-self-immolation cascade to release COS, followed by rapid hydrolysis to H₂S. Contrary to previous reports of similar donors, however, these compounds exhibit significant cytotoxicity and fully inhibited major mitochondrial bioenergetic pathways in bronchial epithelium BEAS2B cells. Similar cytotoxicity profiles were not observed for other H₂S donors, including NaSH, GYY4137, or AP39, at similar concentrations. Furthermore, the analogous CO₂-releasing carbamate control compound was non-cytotoxic, confirming that the observed cytotoxicity or bioenergetics impacts were not due to organic byproducts of donor activation. Taken together, these results led to the hypothesis that the observed effects could be due to a buildup of COS. Supporting this hypothesis, the rate of small ester cleavage by mammalian esterases is likely faster ($\sim 5.1 \times 10^4 - 5.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) than the rate of CA-mediated COS hydrolysis to H₂S,^{22, 24} which would result in a buildup of intracellular COS. Here we extend this hypothesis by preparing a library of esterase-cleaved COS-releasing donors in which the steric bulk of the ester and the electronic properties of the aniline payload are modified. We demonstrate that the differential cytotoxicity of these donors maps to the COS release rates, thus furthering the hypothesis that COS may exert different biological effects than H₂S alone (Figure 1).

RESULTS AND DISCUSSION

To further investigate whether the cytotoxicity of these esterase-activated COS/H₂S donors could be related to COS directly, we chose to probe the relationship between COS release rates and the corresponding cytotoxicity. We hypothesized that if COS buildup was responsible for the observed cytotoxicity, then esters cleaved more quickly should result in increased cell death, whereas esters cleaved more slowly should have a diminished effect. In the esterase-activated donors, the rate of COS release depends not only on the rate of ester cleavage (“triggering”), but also on the rate of self-immolative decomposition. There have been a number of reports demonstrating that rate of esterase activity varies directly with the steric bulk of the ester being cleaved,^{25–26} providing a rational strategy for manipulating the rate of triggering by intracellular esterases. Similarly, recent work has demonstrated that the electronics of the amine payload can affect the rate of thiocarbamate self-immolation.^{27–28}

To probe the effects of steric bulk on the rate of COS release and cytotoxicity of esterase-triggered COS donors, we prepared a library of thiocarbamates functionalized with different esters. To prepare the donors, we first treated 4-hydroxy benzyl alcohol with different alkyl and aryl carbonyl chlorides to afford the corresponding esters. Reaction with *p*-tolyl isothiocyanate furnished the desired thiocarbamates (**TCM1** to **TCM9**) in 14–90% yield (Figure 2a). In parallel, we also prepared the carbamate control compounds, which release CO₂ rather than COS, by treatment of the carbonyl chloride intermediates with *p*-tolyl isocyanate (Figure 2b). To investigate the role of electronic modulation of the aniline payload on the rate of self-immolation and cytotoxicity of these compounds, a similar synthetic sequence was followed to access esterase-triggered COS donors with electron-rich and electron-deficient amine payloads, (**TCM10** to **TCM14**).

With the library of esterase-activated COS/H₂S donors in hand, we next measured the H₂S release from these compounds in the presence of CA (Isozyme II from bovine erythrocytes) and porcine liver esterase (PLE). Direct detection of H₂S using a sulfide-selective electrode is simple and fast, but analogous methods do not exist for rapid COS detection directly in solution. For this reason, we added excess CA to these experiments to ensure no buildup of COS and used the detection of H₂S as an indirect measurement of COS release. We treated compounds **TCM1** – **TCM9** (Figure 2b, left) and **TCM10** – **TCM14** (Figure 2b, right) with 5 U/mL PLE in the presence of CA (25 µg/mL) in PBS buffer (pH 7.4) and observed H₂S release from each of compounds using a H₂S-sensitive electrode. These data confirm that physiologically relevant amounts of CA and PLE are sufficient to result in H₂S release from each of these donors. Consistent with our expectation that steric changes to the esters would result in different cleavage rates, we observed significantly different H₂S release rates and efficiencies from the donor compounds containing a variety of different ester groups (Figure 3a). For example, donors with bulkier esters (cyclohexyl (**TCM7**), adamantyl (**TCM8**), or naphthyl (**TCM9**), yellow, orange, and light blue traces, respectively) generated H₂S more slowly than those with smaller esters (methyl (**TCM1**), *t*-butyl (**TCM5**), or methyl cyclopropyl (**TCM4**), dark green, grey, and magenta traces, respectively). This qualitatively confirms that donors containing larger ester groups produce COS/H₂S more slowly, consistent with slower hydrolysis by PLE.

H₂S release kinetics were also compared for a library of *t*-butyl ester functionalized donors containing a variety of electronically modulated amine payloads. We hoped to systematically decrease the rate of COS release through the introduction of electron-donating groups, although acidification of the N-H proton of the thiocarbamate has been reported to decrease the rate of COS release from similar donors functionalized with electron-withdrawing groups as well.²⁷ Consistent with this hypothesis, the introduction of either strongly electron-withdrawing (NO₂ (**TCM14**), CF₃ (**TCM13**), pink and purple traces, respectively) or electron-donating (Ph (**TCM10**), black trace) groups decreased the rate and efficiency of the donors, indicating that both electron-withdrawing and electron-donating groups slow down the rate of self-immolation following esterase hydrolysis. The donors containing weakly electronically modified amine payloads **TCM5**, **TCM11**, and **TCM12** appear to have very similar initial rates (Figure 3b).

We next sought to determine whether the observed differences in COS/H₂S release rates translated to differences in cytotoxicities of compounds **TCM1** – **TCM14**. To probe these effects, we incubated HeLa cells with the COS donor compounds at 10, 25, 50, and 100 μM for 90 minutes and measured the resultant cell viability against the vehicle using the formazan dye-based CCK-8 cytotoxicity assay. We found that the cytotoxicity of the donors increased as the size of the ester decreased (Figure 3c), with the smallest ester (Me, **TCM1**) resulting in about 70% cell death at 100 μM. No significant cell death was observed, however, when cells were incubated with 100 μM of **TCM9**, which requires hydrolysis of a much larger naphthyl ester and has a much slower rate of COS/H₂S release. To confirm that the observed cytotoxicity was not due to the organic byproducts of the donor constructs after activation, we also investigated the cytotoxicity of the corresponding carbamate control compounds (**CM1** – **CM14**) using the same conditions. Overall, we found significantly less cytotoxicity of all of the carbamates up to 100 μM. No significant trend was observed in the cytotoxicity of the donors as the electronics of the payload were changed. While many of these donors (**TCM5**, **TCM11** – **TCM14**) were cytotoxic, even as low as 25 μM, we did not find any correlation between cytotoxicity and the electronics of the amine payload (Figure 3d). Since the mechanism of decomposition of these donors may change due to acidification of the N-H proton, the cytotoxicity likely does not correspond to the rate of COS production.

This work provides evidence that cellular accumulation of COS is cytotoxic. Importantly, the cytotoxicity observed from many of these COS donors was completely eliminated when HeLa cells were incubated with the analogous, CO₂-releasing carbamates, which control for all other byproducts, suggesting that COS may be directly responsible for the observed effects. Cell death is dose-dependent for all of the cytotoxic COS-releasing compounds, and in general, the most cytotoxic donors were also found to have the most rapid kinetics of H₂S release in the presence of CA (Figure 3e and 3f). Overall, the hypothesis that the inclusion of a larger ester on these donors would decrease the rate of hydrolysis and prevent the build-up of COS was found to hold true. We were not able to systematically decrease the rate of COS release through electronic modulation of the amine payloads, but did find that both strongly electron-withdrawing and electron-donating groups diminished COS/H₂S release, consistent with two previously suggested effects: that electron-donating groups decrease the rate of self-immolation, and electron-withdrawing groups can result in a change

in the mechanism of H₂S release due to acidification of the N-H proton on the thiocarbamate.²⁷ Due to a potential change in mechanism supported by a decrease in H₂S production from donors containing electron-withdrawing payloads, it is impossible to correlate the cytotoxicity of these particular donors with their rate of COS/H₂S release.

In addition to providing new insights into the differential impacts of COS and H₂S, this work also increases the tools available for increasing basal H₂S concentrations without the need for external triggering mechanisms or consumption of cellular nucleophiles. To confirm that these donors release COS/H₂S in a cellular environment, we incubated 100 μM Cy-TCM (TCM7) with SF7-AM in HeLa cells and observed an increase in fluorescence corresponding to H₂S donation from the scaffold (Figure 4).²⁹ This confirms the basic cellular viability of this compound as an H₂S donor. Although the faster-releasing donors are too cytotoxic for use as efficacious H₂S donors, the slower-releasing donors provide a library of enzyme-activated COS/H₂S donors viable for use in cell-based experiments.

In conclusion, this work supports the hypothesis that rapid accumulation of COS likely results in cytotoxicity.²³ Conclusively disentangling the effects of COS delivery from the physiological effects of H₂S will require a systematic study of COS, the various CA isoforms, and the potential for subcellular localization of COS delivery from various donors. The work reported here suggests the likely role of COS in the cytotoxicity of many of these compounds and provides an important piece of early evidence that COS delivery may produce a cellular response that is different than that observed from H₂S alone.

METHODS

General Materials and Methods.

Reagents were purchased from Sigma-Aldrich or Tokyo Chemical Industry (TCI) and used as received. SF7-AM was synthesized as previously reported.³⁰ Spectroscopic grade, inhibitor-free THF was deoxygenated by sparging with argon followed by passage through a Pure Process Technologies solvent purification system to remove water. Deuterated solvents were purchased from Cambridge Isotope Laboratories and used as received. Silica gel (SiliaFlash F60, Silicycle, 230–400 mesh) was used for column chromatography. ¹H, ¹³C{¹H}, and ¹⁹F NMR spectra were recorded on a Bruker 500 or 600 MHz instrument (as indicated). Chemical shifts are reported in ppm relative to residual protic solvent resonances. Mass spectrometric measurements were performed on a Xevo Waters ESI LC/MS instrument or by the University of Illinois, Urbana Champaign MS facility. H₂S electrode data were acquired with a Unisense H₂S Microsensor Sulf-100 connected to a Unisense Microsensor Multimeter. All air-free manipulations were performed under an inert atmosphere using standard Schlenk techniques or an Innovative Atmospheres N₂-filled glove box. HeLa cells were purchased from ATCC (Manassas, Virginia, USA). Cell imaging experiments were performed on a Leica DMI8 fluorescence microscope, equipped with an Andor Zyla 4.2+ sCMOS detector. Fluorescence intensity measurements were calculated using Fiji (ImageJ).³¹ Fluorescence intensities were measured in Fiji, with images scaled to 32-bit, and the error is reported as the standard mean error.

H₂S Electrode Experiments.

Scintillation vials containing 20.00 mL of phosphate buffer (140 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) were prepared in an N₂-filled glovebox. The Unisense electrode was inserted into the vial and the vial was capped with a split-top septum to minimize oxidation. The current was allowed to equilibrate prior to starting the experiment. With moderate stirring, the CA stock solution (50 μ L, CAII from Bovine Erythrocytes) was injected, followed by subsequent injections of TCM stock solution (50 μ L) and PLE stock solution (100 μ L). H₂S release was monitored until leveling off.

CCK-8 Cell Viability Experiments.

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO₂. 96-well plates were seeded with 15,000 cells/well overnight then washed, incubated in FBS-free DMEM containing vehicle (0.5% DMSO), TCA (10100 μ M), or carbamate (10100 μ M) for 90 minutes. Cells were then washed with PBS and CCK-8 solution (1:10 in FBS-free DMEM) was added to each well, and cells were incubated for 1–2 hours at 37 °C under 5% CO₂. The absorbance at 450 nm was measured using a microplate reader and the cell viability was measured and normalized to the vehicle group. Results are expressed as mean $\pm$ SD (n=6). *P* values were calculated using a Student's T-test in Excel compared to DMSO alone.

Cell Imaging.

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO₂. Imaging dishes were seeded with HeLa cells overnight and then washed and incubated with SF7-AM (5 μ M) and Hoechst 33342 (5 μ g/mL) in FBS-free DMEM for 30 min. Cells were then washed with PBS and incubated with either Cy-TCM (100 μ M) or vehicle (DMSO, 0.5%) in FBS-free DMEM for 60 minutes prior to being washed with PBS and imaged. Imaging was performed once, and the fluorescence intensities were calculated from the images shown, with 77 cells in the TCM images and 116 cells in the control.

Synthesis

General procedure for the synthesis of phenol esters.—4-Hydroxy benzyl alcohol (1.0 equiv.) was dissolved in anhydrous THF (0.1 M solution), under and atmosphere of N₂. The solution was cooled to 0 °C, followed by addition of Et₃N. The reaction mixture was let stir for 5 minutes, after which the carbonyl chloride was added dropwise over 20 minutes. The resultant mixture was stirred at 0 °C until the completion of the reaction indicated by TLC. The reaction was quenched by adding brine (30 mL), and the aqueous solution was extracted with ethyl acetate (3 $\times$ 20 mL). The organic layers were combined, dried over anhydrous MgSO₄, and concentrated under reduced pressure. The crude product was purified by silica column chromatography. Full spectroscopic data for each compound is reported in the SI. The preparation of MeCp-OH is also reported in the SI.

General procedure for preparation of thiocarbamates.—The functionalized benzyl alcohol (1.0 equiv.) was dissolved in anhydrous THF (0.2 M solution) under an atmosphere of N₂. Aryl isothiocyanate (1.1 equiv.) was added, followed by DBU (1.25 equiv.) at 0 °C. The resultant mixture was warmed to rt and stirred monitored by TLC. The reaction was quenched upon observation of by-product formation by TLC by addition of brine (20 mL), and extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over anhydrous MgSO₄ or Na₂SO₄, concentrated under reduced pressure, and purified by silica column chromatography. Full spectroscopic data for each compound is reported in the SI.

General procedure for preparation of carbamate controls.—Functionalized benzyl alcohol (1.0 equiv.) was dissolved in anhydrous THF (0.1 M solution) under an atmosphere of N₂. Aryl isocyanate (0.90 equiv.) was added, followed by DBU (1.25 equiv.) at 0 °C. The resultant mixture was warmed to rt and stirred monitored by TLC. The reaction was quenched upon observation of by-product formation by TLC by addition of brine (20 mL), and extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over anhydrous MgSO₄, concentrated under reduced pressure, and purified by silica column chromatography. Full spectroscopic data for each compound is reported in the SI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS.

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Previous work: Pluth, 2017 **This work:** **Figure 1.**

Esterase-triggered thiocarbamate-based H₂S donors exhibit increased cytotoxicity, potentially due to the buildup of intracellular COS.

Figure 2.

(a) Synthetic scheme for the development of a library of esterase-activated thiocarbamate COS/H₂S donors (**TCM1–14**) and (b) table showing all compounds used in this study (**TCM1–14** and **CM1–14**) with yields.

Figure 3.

H₂S release curves for compounds (a) TCM1 – TCM9 and (b) TCM5, TCM10-TCM14) in the presence of PLE (5 U/mL) and CA (25 μ g/mL) at pH 7.4. (c and d) Cytotoxicity of compounds in HeLa cells. Data for donors (TCM1 – TCM14) is shown for 10 – 100 μ M and compared to the cytotoxicity of the carbamate control compounds (CM1 – CM14) at 100 μ M. (c) Cytotoxicity data for donors containing varying ester groups (TCM1 – TCM9), with steric bulk of the ester group decreasing from left to right. (d) Cytotoxicity data for donors containing varying amine payloads (TCM5, TCM10 – TCM14), with electron donating-ability of the payload decreasing from left to right. Results are expressed as mean $\pm$ SD ($n=6$). The values that are significantly different by Student's t test are indicated by asterisks as follows: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. (e) Dual-axis comparison of cytotoxicity of various ester-containing donors at 100 μ M and the rate of H₂S release from these

compounds in the presence of PLE and CA. (f) Table of the rates of H₂S release and percent cell viability of various ester-containing donors at 100 μ M.

Figure 4.

(a) Live-cell imaging of H₂S release from TCM7 in HeLa cells. HeLa cells were treated with SF7-AM (5 μ M) and Hoechst (5 μ g/mL) for 30 min, washed, and incubated with FBS-free DMEM containing TCM7 (100 μ M, bottom) or DMSO (0.5%, top) for one hour. Cells were then washed and imaged in PBS. Scale bar = 100 μ m. (b) Relative integrated fluorescence intensity of cells treated with TCM7 versus vehicle treated cells.