ACES Asian Chemical Editorial Society

DOI: 10.1002/asia.201901148

CHEMISTRY AN ASIAN JOURNAL Full Paper

VIP

Special Issue

Carbonyl Sulfide (COS) Donor Induced Protein Persulfidation Protects against Oxidative Stress

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Abstract: The emergence of hydrogen sulfide (H_2S) as an important signalling molecule in redox biology with therapeutic potential has triggered interest in generating this molecule within cells. One strategy that has been proposed is to use carbonyl sulfide (COS) as a surrogate for hydrogen sulfide. Small molecules that generate COS have been shown to produce hydrogen sulfide in the presence of carbonic anhydrase, a widely prevalent enzyme. However, other studies have indicated that COS may have biological effects which are distinct from H_2S. Thus, it would be useful to develop tools to compare (and contrast) effects of COS and H_2S. Here we report enzyme-activated COS donors that are capable of inducing protein persulfidation, which is symptomatic of generation of hydrogen sulfide. The COS donors are also capable of mitigating stress induced by elevated reactive oxygen species. Together, our data suggests that the effects of COS parallel that of hydrogen sulfide, laying the foundation for further development of these donors as possible therapeutic agents.

Introduction

Carbonyl Sulfide (COS) is a major constituent of the earth's atmosphere and forms an important part of the global sulfur cycle. COS originates from volcanoes or hot springs and this gas has been suggested as a possible chemical component during origin of life. COS is also a source of energy for certain bacteria. Thiocyanate hydrolases are a class of COS synthesizing enzymes that are found in bacteria, however, its production in mammalian cells still remains elusive.[1] Since COS is rapidly hydrolyzed to hydrogen sulfide (H_2S) in the presence of carbonic anhydrase (CA),[2] a widely prevalent enzyme, this gas has been utilized as a surrogate for enhancing H_2S within cells. Small molecules that generate COS, known as COS donors,[3] including those activatable by an enzyme,[4] light,[5] nucleophiles,[6] click chemistry[7] or elevated hydrogen peroxide[8] are in development. Some have shown potential cytoprotective effects and even endothelial proliferative effects. These studies have naturally assumed that any physiological effects demonstrated by the COS donor is due to H_2S.[6b,8b] However, recent reports raised a possibility that COS may be a potential biomolecule with effects distinct from H_2S. Since certain COS donors are associated with trigger-independent decomposition pathways, a new class of donors is necessary.[4b] The new donor needs to be selective, and the byproducts of activation need to be tolerated well by cells. This would allow for systematically studying the effects of COS in cells and compare (and contrast) its effects with hydrogen sulfide. Here we report the development of enzyme activated COS donors that are highly suited for interrogating the effects of this gas and we demonstrate that COS donors induce persulfidation in cells that may contribute to its observed cytoprotective effects.

Due to the in-built catalytic efficiency of an enzyme, an enzyme-prodrug approach was used to design new COS donors. Since the effects of H_2S are dependent on its concentration as well as rate of generation, any donor must have structural features that allow for rates to be modulated. Based on the aforementioned criteria, NAD(P)H quinoneoxidoreductase 1 (NQO1) also referred to as DT-Diaphorase, which is predominantly a two electron reducing cytosolic enzyme used by the cell for reduction of quinones to hydroquinones, was chosen as the prodrug-metabolizing enzyme.[9] Immunohistochemical analysis of NQO1 revealed its expression in certain tissues including epithelial cells in the colon.[10] Increased expression of this enzyme under oxidative stress and electrophilic stress conditions, likely due to its role in removal of xenobiotics, is also reported.[11] NQO1 has been used for delivering drugs or fluorophores (for imaging).[9a,12,13] Since H_2S has been shown to play an important protective role in the colon as well as under oxidative stress conditions, we designed a NQO1-activable COS donor (Figure 1b). This compound is expected to undergo reduction to produce a hydroquinone inter-

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This manuscript is part of a special issue celebrating the 20th anniversary of the Chemical Research Society of India (CRSI). A link to the Table of Contents of the special issue will appear here when the complete issue is published.

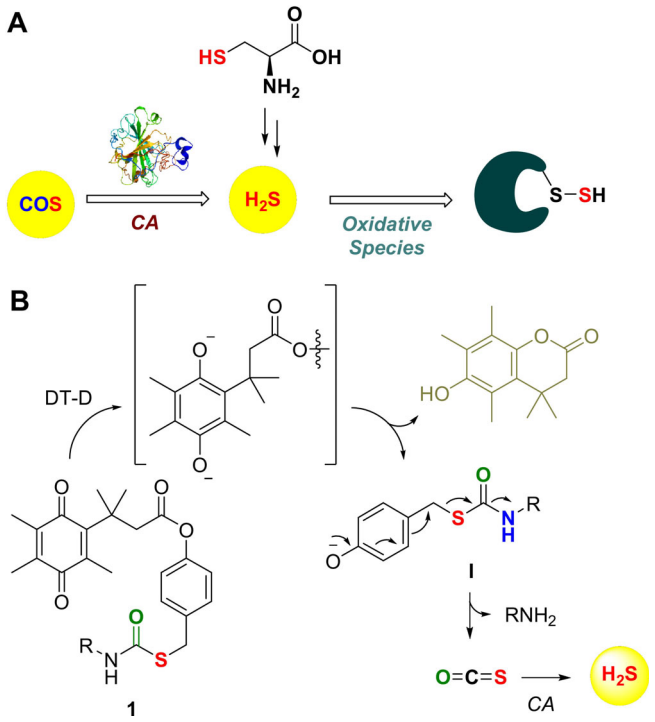
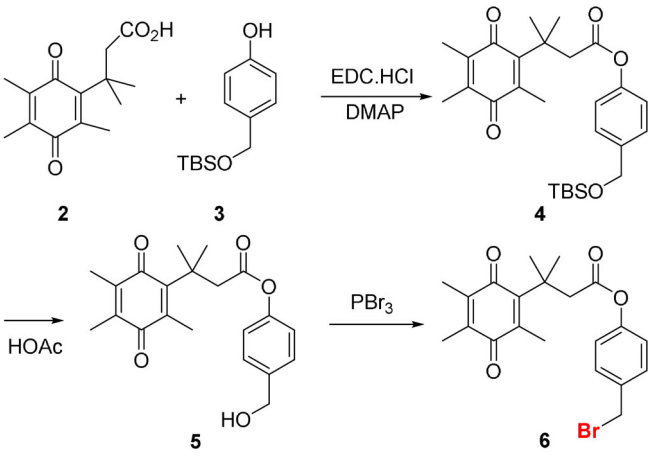
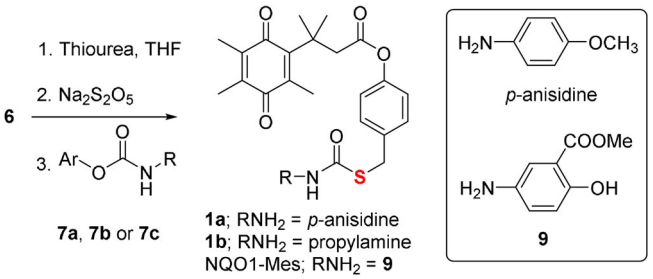


Figure 1. a) Hydrolysis of COS in the presence of carbonic anhydrase (CA) produces hydrogen sulfide (H_2S). The major biosynthetic pathway for H_2S generation include metabolism of cysteine. In the presence of elevated oxidative species, cysteine-containing proteins are persulfidated, that is, modified to their persulfides. b) Design of a bioreductively activated COS donor. NAD(P)H quinoneoxidoreductase 1 (NQO1), also referred to as DT-Diaphorase (DT-D) reduces the quinone to a diolate intermediate, which rapidly produces a lactone. The intermediate I, which has been previously shown to be produced by other methods rearranges by self-immolation to produce COS, which in the presence of CA produces hydrogen sulfide. The rate of hydrogen sulfide production may depend on the basicity of the amine.



Scheme 1. Synthesis of NQO1 triggered COS/H_2S donors.



Scheme 2. Synthesis of NQO1 triggered COS/H_2S donors. Inset, aromatic amines used in this synthesis. For structures of **7a**–**7c**, please see SI (Figure S1).

mediate, which would lactonize to produce intermediate I. Our lab recently reported that rates of release of COS could be modulated by varying the basicity of the amine.[8a] Here, the suggested intermediate I self-immolates to produce COS and an amine (Figure 1b). COS is hydrolysed to H_2S in the presence of CA.

Results and Discussion

Since our previous data suggested that the rate of H_2S generation by the aniline-derivative was significantly higher when compared with an aliphatic amine-based donor, we decided to synthesize **1a**, a *p*-anisidine-based donor and **1b**, with propylamine as a leaving group. Compound **6** was first synthesized in three steps starting from compound **2** (Scheme 1). Compound **6** was then reacted with thiourea for 12 h to form a thiourea adduct which was further hydrolysed to give thiol. The thiol so obtained was found to be unstable and therefore was directly taken to the next step without further purification to react with respective carbamates and give compound **1a** in 65% and **1b** in 34% yield (Scheme 2). Compound **5** was used as a negative control in further experiments.

First, the H_2S releasing capability of the molecules in the presence of NQO1 was evaluated using a methylene blue formation assay.[14] Compounds were incubated in the presence of NQO1, NADH and CA in phosphate buffer (pH 7.4) at 37 °C. At pre-determined time points, an aliquot of the reaction mixture was taken, treated with the methylene blue cocktail and further incubated to allow the formation of the complex. An absorbance profile was measured to follow the formation of methylene blue complex. A time dependent increase in the absorbance profile for compound **1a** was observed over a period of 4 h (Figure 2a). The kinetics of H_2S release was measured for both the compounds under similar conditions. The rate constants for compounds **1a** and **1b** were calculated by fitting the initial rate data to first order kinetics. The rate constants for compound **1a** and **1b** were calculated to be 0.016 min^{-1} and 0.008 min^{-1} respectively (See SI, Figure S2). This trend is in accordance with our previous reports, although the magnitude of difference between the fast and slow donor was somewhat diminished possibly due to the poor solubility of the donors.[8a]

Next, the decomposition of **1a** in the presence of NQO1 was monitored by HPLC analysis. Compound **1a** when incubated in buffer was eluted at a retention time of 16.6 min (See SI, Figure S5a). Upon treatment with NQO1 in phosphate buffer (pH 7.4) containing NADH at 37 °C, we observed a complete disappearance of the peak for **1a** within 30 min of incubation. This was in accordance with the previous studies, indicative of

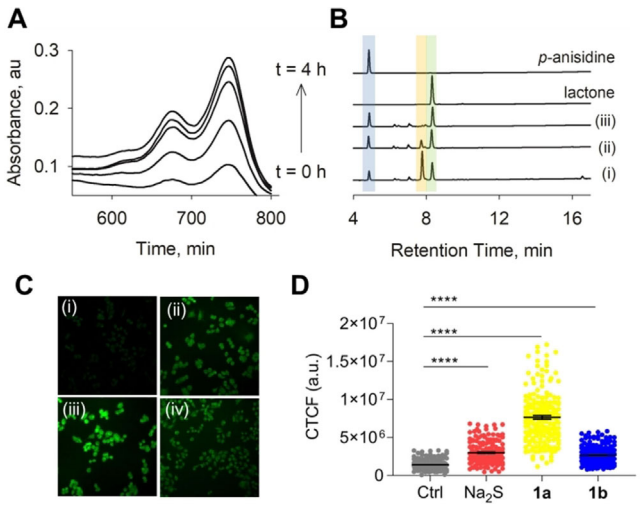


Figure 2. a) Representative methylene blue formation plots determined by spectophotometry after incubation of **1 a** (50 μM) in the presence of NQO1 (10 μg mL^{-1}), NADH (250 μM) and CA in phosphate buffer (pH 7.4). b) Representative HPLC traces for decomposition of compound **1 a**, (i) 30 min (ii) 1 h (iii) 2 h. c) Representative images of persulfidation induced by NQO1 responsive H_2S donors (**1 a** and **1 b**) within human colon carcinoma (DLD-1) cells ($n=3$). Scale bar is 50 μm. Increase in the persulfidation signal was observed with the H_2S donors, (i) ctrl represents untreated cells (ii) Na_2S (200 μM) (iii) **1 a** (10 μM) (iv) **1 b** (10 μM). d) Quantification of the persulfidation signal observed by the H_2S donors ($n=3$).

the rapid reduction of the quinone in the presence of NQO1 enzyme; the peak at 8.3 min corresponded to the formation of lactone (Figure 2 b).[15] The rate of decomposition of the compound was calculated to be 0.063 min^{-1}which correlated well with the rate of formation of lactone that is, 0.066 min^{-1}(See SI, Figure S5 c,d). Another peak corresponding to the formation of an intermediate was observed at 7.8 min which decomposed over a period of 2 h with concomitant formation of *p*-anisidine at 4.8 min (See ESI, Figure S5 b). The results obtained support our hypothesis that the quinone is reduced by NQO1 to form hydroxyquinone (Figure 1 b), which immediately undergoes intramolecular cyclization to generate intermediate I. The intermediate thus formed decomposes over a period of 2 h to release COS which is then hydrolyzed to H_2S by CA.

Having established the in vitro release of H_2S from the donor motifs, we next investigated the cellular aspects of controlled generation of this reactive gaseous species. Protein persulfidation is a protective post-translational modification associated with H_2S wherein, thiol of a cysteine (Cys-SH) reacts with H_2S in the presence of oxidants to form more reactive persulfide (Cys-SSH).[16] Due to the inherent lack of stability of persulfides, detecting these transient species is challenging. We used an improved tag switch technique to detect persulfides in cells.[17] NQO1 enzyme is overexpressed in colon cancers which include human colon adenocarcinoma, DLD-1 cells.[18] Thus, in order to test the persulfidating capability of the donors, DLD-1 cells were treated with H_2S donors (10 μM) for 1 h to induce persulfidation. Cells were further treated with methyl sulfonylbenzathiazole (MSBT), an aromatic thiol blocking agent, to block the persulfides in the form of mixed disulfides. This was followed by treatment with CN-BOT; a nucleophile tagged to a fluorescent reporter, to tag protein persulfides. The cells were then imaged under a fluorescence microscope in FITC channel. A significant enhancement in the fluorescence signal was observed with **1 a** compared to the untreated control, indicative of the protein persulfidation induced by **1 a**. A diminished fluorescence signal was observed with compound **1 b** which could be associated to the lower rate of H_2S release observed from **1 b** (Figure 2 c). Thus, the results obtained suggest that the extent of persulfidation induced by H_2S depends on the rate of its release. The observed fluorescence signal for protein persulfidation was quantified and plotted as corrected total cell fluorescence (CTCF, a.u.) (Figure 2 d). Na_2S showed a diminished fluorescence signal compared to compound **1 a** which could be due to the low effective concentration of H_2S obtained from the aqueous solution of Na_2S. Hence, COS donor **1 a** is able to permeate cells to induce protein persulfidation.

The cytotoxicity profile of the molecules was assessed using a standard cell viability assay. DLD-1 cells were treated with varying concentration of compounds for 24 h following which the cell viability was monitored. The compounds were found to be well tolerated by the cells at 50 μM concentration (See SI, Figure S12 a,c). Human breast cancer, MCF-7 cells were next incubated with varying concentrations of **1 a** and cell viability was measured after 72 h of incubation. No significant decrease in viable cells was seen, suggesting that the reported scaffolds are non-toxic (See SI, Figure S9). The obtained results were further confirmed by using LDH release assay. Here, MCF-7 cells were treated with varying concentrations of **1 a** and after 24 h measurements revealed that this compound was well tolerated at 50 μM concentration (See SI, Figure S10). This result is in contrast to recent reports that suggest that fast COS donors are associated with cytotoxicity and could be related, in part, to non-specific decomposition pathways associated with the previously reported donors.[4b,c]

The donors reported herein were non-toxic and capable of inducing protein persulfidation within cells. This led us to further investigate the cytoprotective effects of these donors, against oxidative stress induced toxicity. JCHD is a cell-permeable small molecule superoxide generator; since elevated ROS is cytotoxic, this is a good tool to study oxidative stress (See SI, Figure S11).[19] When cells were co-treated with JCHD and **1 a**, a dose-dependent increase in the cell viability was observed. However, no increase in the cell viability was observed with compound **1 b**, which is consistent with the poor capability for this compound to induce protein persulfidation (See SI, Figure S12 b,d). It appears that persulfidation by the COS donor may determine the cytoprotective effects of H_2S.

To further validate the hypothesis and evaluate the cytoprotective effects of H_2S, we synthesized a H_2S-NSAID hybrid donor and studied its effects. Mesalamine is used as first in line therapy for the treatment of colitis.[20] But, the drug is effective only against mild to moderate forms of this disease and lacks potency to treat severe form of colitis. Wallace and co-workers in 2007 showed that ATB-429, a H_2S-mesalamine hybrid, exhibited marked increase in anti-inflammatory properties, thereby increasing the effectiveness and potency of the drug in a

murine model of colitis.[21] Therefore, we proceeded to investigate the cytoprotective effects of these donors against xenobiotic induced stress in human colon carcinoma, DLD-1 cells. We synthesized NQO1 activated COS-Mesalamine donor, NQO1-Mes, using the above mentioned protocol in 43% yield (Scheme 2). This compound was found to generate H_2S (electrode data, Figure 3a; methylene blue data, Figure 3b). In the presence of a known inhibitor for DT-D, diminished H_2S production was observed. HPLC analysis showed complete decomposition of this compound to produce methylester derivative of mesalamine, **9** (See SI, Figure S6). The rate constant for hydrogen sulfide release was calculated to be 0.02 min^{-1} (see SI, Figure S3). This donor was also found to induce persulfidation in cells (Figure 3c,d) and was able to protect from JCHD-induced cytotoxicity (Figure 3f). Mesalamine alone was not found to be as effective possibly due to poor cell permeability. The cell type-specificity of the donors was assessed by testing

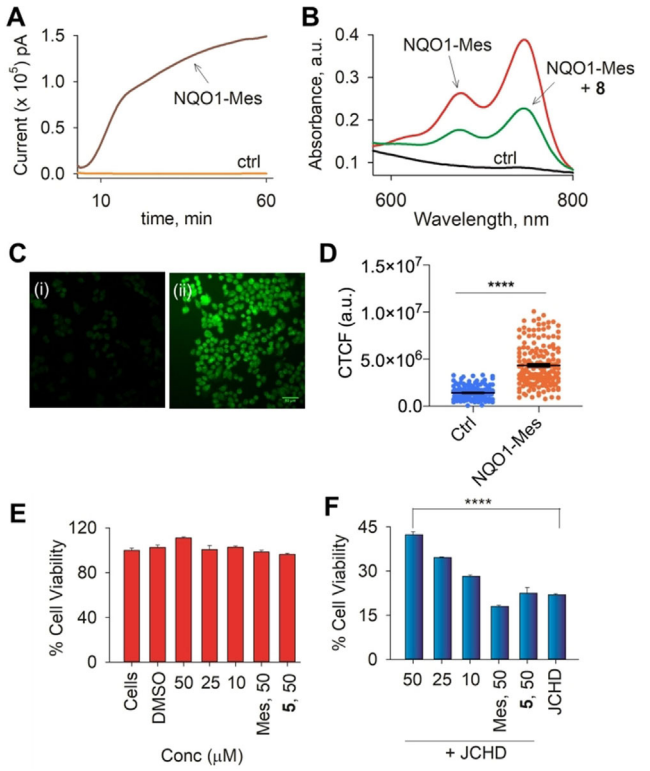


Figure 3. a) Representative trace for H_2S release from NQO1-Mes (50 μM) in the presence of NQO1 (10 μg mL^{-1}), NADH (250 μM) and CA in PBS (pH 7.4, 50 mM) using sulfide selective electrode. Ctrl represents NQO1-Mes (50 μM) alone in media containing 10% FBS. No H_2S was produced from the compound in media. b) Methylene blue formation determined by spectophotometry after incubation of NQO1-Mes(100 μM) in the presence of NQO1, NADH and CA. Diminished signal was observed in the presence of dicoumarol **8**, a known inhibitor of NQO1. c) Representative images of persulfidation induced by NQO1-Mes in human colon carcinoma (DLD-1) cells ($n=3$). Scale bar is 50 μm. (i) ctrl (ii) NQO1-Mes (10 μM). d) Quantification of the persulfidation signal observed by the H_2S donors ($n=3$). e) Cell viability for NQO1-Mes in DLD-1 cells. f) Cytoprotective effects of NQO1-Mes against JCHD (15 μM) induced oxidative stress in DLD-1 cells over a period of 24 h. Results are expressed as Mean ± SEM ($n=3$). [Legend: **** implies $p<0.0001$ vs. JCHD]. Compound **5** (50 μM) represents the negative control and Mes represents mesalamine (50 μM). No significant increase in the cell viability was observed during incubation of **5** and mesalamine with JCHD.

the cytoprotective effects of NQO1-Mes in mouse embryonic fibroblast, WT-MEF cells, reported previously to have low expression levels of NQO1 enzyme.[22] The cytoprotective effects were tested using the aforementioned protocol. WT-MEF cells were co-treated with increasing concentrations of H_2S donors and JCHD (15 μM) for 24 h following which the cell viability was monitored. As expected, no significant increase in cell viability was observed with the H_2S donors compared to the JCHD control (See SI, Figure S13, S14). The results indicate that the H_2S donors reported herein are selective towards activation by NQO1 enzyme.

As COS has developed into an important delivery tool for localizing H_2S, it is natural to assume that the physiological effects observed with the reported COS donors are attributable to H_2S. But recent advancements in the field have raised the possibility of COS acting as a potential gaseous signalling molecule acting distinctly from H_2S; thus posing an important question on the mechanism of action of COS within cells. We worked towards addressing this ambiguity by synthesizing NQO1 activated COS donors which are selective, capable of modulating the rate of COS generation with release of potentially innocuous byproducts. It is hypothesized that NQO1 along with NADH cofactor reduces the quinone moiety in compound **1** to hydroquinone which undergoes immediate cyclisation to subsequently release lactone and intermediate I. The intermediate thus formed releases COS depending on the basicity of the leaving group amine. COS released is readily hydrolyzed to H_2S in the presence of CA which is assayed by various independent techniques. Therefore, the donors were found to release H_2S in the presence of NQO1 and CA. Modulating the release of COS/H_2S proved to be an important tool to delineate the mechanism of action of COS within cells.

Protein persulfidation is a characteristic post translational modification associated with H_2S.[23] Protein persulfides, thus assume importance in sulfur mediated signaling and therapeutics. Strategies to release small molecule persulfides have been developed which include—spontaneous persulfide donors,[24] enzyme activated donors,[25] oxidative stress induced persulfide release,[19b,26] pH or F^- dependent persulfide release.[27] These donors provide testament to therapeutic utility of enhancing persulfidation levels within cells. Therefore, we investigated if the donors reported herein get activated by NQO1 present within cells to release H_2S and further induce protein persulfidation. The persulfides can be trapped and detected using improved tag switch technique which is widely accepted for detection of persulfides in cells.[17] Compounds **1a** and **1b** significantly enhanced the persulfidation levels in DLD-1 cells which is indicative of the release of H_2S within cells. However, the extent of persulfidation induced by the donors was found to be dependent on the rate of release. The result indicates that COS produced in cells is hydrolyzed to generate H_2S which induces protein persulfidation depending on the rate of release; therefore, providing an important link towards understanding the mechanism of COS activity. Finally, to investigate if the observed differences could be translated to mediate the physiological effects within cells, we tested the cytoprotective effects of the donors against xenobiotic induced stress. Human colon

adenocarcinoma, DLD-1 cells were treated with oxidative stress inducing agent (JCHD) to induce cell death. When co-treated with COS donors we observed that compound **1a** showed significant enhancement in viable cells compared to compound **1b** which is likely due to the difference in their persulfidation capability. The results obtained were further validated by NQO1 activated COS-NSAID hybrid donor which showed cytoprotective effects against oxidative stress. This is again consistent with the behavior of COS much like H_2S.

Taken together, the effects of COS generated by this class of donors is protein persulfidation, which is symptomatic of intermediacy of hydrogen sulfide. The donors themselves as well as their byproducts were well tolerated and the NSAID-hybrid was able to protect cells from oxidative stress supporting the further development of this class of compounds. These tools lay the foundation for systematically studying the redox biology of COS, a newly emergent bioactive gaseous molecule with high therapeutic potential.

Conclusions

While the geological COS production and as a consequence exposure to this gas is not in doubt, whether COS is biosynthesized remains to be determined. COS has been suggested as a stable reservoir for hydrogen sulfide and other reports indicate distinct effects of COS when compared with H_2S. Since careful generation of COS with relatively innocuous byproducts is crucial to delineate such effects, the tools that we have developed are important. Our study demonstrates that COS donors are well tolerated by cells and induce protein persulfidation in cells. The donors were also found to protect cells from oxidative stress, which is symptomatic of H_2S generation. Together, our study suggests a link between COS and persulfidation supporting a significant role for H_2S in COS biology.

Experimental Section

All reactions were conducted under nitrogen atmosphere. All the chemicals were purchased from Sigma Aldrich. The solvents were purchased from commercial sources and used as received unless stated otherwise. Column chromatography was performed using silica gel-Rankem (60- 120 mesh) or silica gel Spectrochem (100–200 mesh) as the stationary phase. Preparative high-performance liquid chromatography (HPLC) was done using Combiflash EZ prep UV using a Kromasil®C-18 preparative column (250 mm × 21.2 mm, 5 μm). ^{1}H and ^{13}C spectra were recorded on a JEOL 400 MHz (or 100 MHz for ^{13}C) or a Bruker 400 MHz (or 100 MHz for ^{13}C) spectrometer unless otherwise specified using either residual solvent signals ($CDCl_3$ δH = 7.26 ppm, δC = 77.2 ppm) or as an internal tetramethylsilane (δH = 0.00, δC = 0.0). Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The following abbreviations are used: bs (broad signal), m (multiplet), s (singlet), d (doublet), t (triplet) and dd (doublet of doublets). High-resolution mass spectra were obtained from HRMS-ESI-Q-Time of Flight LC/MS. FT-IR spectra were recorded using BRUKER-ALPHA FT-IR spectrometer. Analytical HPLC was performed on an Agilent1260-infinity with Phenomenex®C-18 reverse phase column (250 mm × 4.6 mm, 5 μm). Photometric measurements were performed using a Thermo Scientific Varioskan microtiter plate reader. Compounds **2**, **3**, **7a**–**7c** were synthesized using previously reported procedures and were taken forward without purification.[8a, 15b]

Synthesis of 4-(((tert-butyldimethylsilyl)oxy)methyl)phenyl 3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanoate (4): To a solution of **2** (1.22 g, 4.89 mmol) and **3** (0.8 g, 4.07 mmol) in dry DCM at 0 °C, EDC.HCl (1.56 g, 8.15 mmol) and DMAP (1 g, 8.15 mmol) were added. The reaction mixture was stirred at 0 °C. The progress of the reaction was monitored by TLC. The reaction was complete after 10 min following which the reaction mixture was washed with 1 N HCl, organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude was purified using column chromatography to give **4** (1.36 g, 71%) as yellow oily liquid. FT-IR $\tilde{\nu}_{max}$ = 1750, 1644, 1258 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.28 (d, J = 8.7 Hz, 2H), 6.92 (d, J = 8.6 Hz, 2H), 4.69 (s, 2H), 3.23 (s, 2H), 2.17 (s, 3H), 1.93–1.91 (m, 6H), 1.52 (s, 6H), 0.93(s, 9H), 0.08 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 190.9, 187.4, 171.4, 152.0, 149.2, 142.9, 139.1, 139.0, 138.5, 127.0, 121.2, 64.4, 47.7, 38.5, 28.9, 25.9, 18.4, 14.3, 12.6, 12.1, −5.3 ppm; HRMS (ESI-TOF) for $[C_{27}H_{38}O_5Si + H]^+$: calcd 471.2567, found 471.2570.

Synthesis of 4-(hydroxymethyl)phenyl 3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanoate (5): To a solution of **4** (0.69 g, 1.45 mmol) in THF (5 mL), water (5 mL) and acetic acid (15 mL) were added. The progress of the reaction was monitored by TLC. Reaction was complete after 6 h of stirring. The reaction mixture was then quenched with saturated $NaHCO_3$ solution and the aqueous layer was extracted by DCM and dried over Na_2SO_4. The organic layer was concentrated under vacuo. The crude was purified by column chromatography to give **5** (0.5 g, 97%) as yellow liquid. FT-IR $\tilde{\nu}_{max}$ = 3510, 1747, 1643 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.33 (d, J = 8.3 Hz, 2H), 6.95 (d, J = 8.4 Hz, 2H), 4.64 (s, 2H), 3.24 (s, 2H), 2.17 (s, 3H), 1.92–1.91 (m, 6H), 1.52 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 190.9, 187.4, 171.4, 152.0, 149.8, 142.9, 139.1, 138.6, 138.5, 128.0, 121.6, 64.7, 47.7, 38.4, 28.9, 14.4, 12.6, 12.1 ppm; HRMS (ESI-TOF) for $[C_{21}H_{24}O_{5+}H]^+$: calcd 357.1702, found 357.1711.

Synthesis of 4-(bromomethyl)phenyl 3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanoate (6): To a solution of **5** (0.51 g, 1.43 mmol) in dry DCM, tribromophosphine (170 μL, 1.71 mmol) was added at 0 °C. The reaction was stirred and the progress was monitored by TLC. After completion of the reaction in 1 h, the reaction mixture was quenched with saturated solution of $NaHCO_3$ and the aqueous layer was extracted by EtOAc. The combined organic layer was dried over Na_2SO_4 and concentrated under vacuo. Crude was purified by column chromatography and compound was obtained as yellow liquid with 43% yield. FT-IR $\tilde{\nu}_{max}$ = 2924, 2856, 1747, 1648, 1601 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.35 (d, J = 8.5 Hz, 2H), 6.95 (d, J = 8.6 Hz, 2H), 4.45 (s, 2H), 3.24 (s, 2H), 2.17 (s, 3H) 1.93–1.92 (m, 6H), 1.52 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 190.8, 186.7, 171.2, 151.8, 150.3, 142.8, 138.6, 138.2, 135.4, 130.2, 121.9, 47.6, 38.4, 32.6, 28.9, 14.4, 12.7, 12.1 ppm; HRMS (ESI-TOF) for $[C_{21}H_{23}BrO_{4+}H]^+$: calcd 419.0858, found 419.0836.

Synthesis of 4-((((4-methoxyphenyl)carbamoyl)thio)methyl)-phenyl 3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanoate (1a): To a solution of **6** (0.30 g, 0.71 mmol) in dry THF, thiourea (0.11 g, 1.43 mmol) was added at room temperature. The reaction was stirred overnight at room temperature. After completion of the reaction in 12 h, the solvent was removed under vacuum to obtain a yellow coloured solid. The crude was dissolved in 15 mL water and 20 mL DCM under nitrogen atmosphere. $Na_2S_2O_5$ (0.54 g, 2.86 mmol) was then added to the reaction mixture and refluxed at 60 °C. After completion of the reaction in 4 h, the crude was extracted using DCM. The organic layer was dried

over Na_2SO_4 and concentrated under vacuo. The product obtained was unstable and therefore was taken directly to the next step. Crude was dissolved in dry DCM (10 mL) followed by the addition of **7a** (0.34 g, 1.19 mmol) and Et_3N (470 μL, 3.37 mmol). The reaction was stirred at room temperature. The progress of the reaction was monitored TLC. After completion in 2 h, the reaction was quenched by the addition of water and extracted by DCM (20 mL). The crude was purified by preparative HPLC using kromasil C-18 column and water: acetonitrile as mobile phase to give **1a** (241 mg, 65%). FT-IR $\tilde{\nu}_{max}$ = 3336, 1745, 1644 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.33–7.26 (m, 4H), 7.01 (s, 1H), 6.90 (d, J = 8.5 Hz, 2H), 6.85 (d, J = 9 Hz, 2H), 4.15 (s, 2H), 3.78 (s, 3H), 3.22 (s, 2H), 2.17 (s, 3H), 1.92–1.91 (m, 6H), 1.51 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 190.9, 187.4, 171.4, 151.9, 149.5, 142.9, 139.1, 138.6, 135.8, 130.0, 121.6, 114.3, 55.5, 47.7, 38.4, 33.8, 28.9, 14.4, 12.7, 12.1 ppm; HRMS (ESI-TOF) for $[C_{29}H_{31}NO_6S+H]^+$: calcd 522.1950, found 522.1945.

Synthesis of4-(((propylcarbamoyl)thio)methyl)phenyl 3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanoate (1b): Compound **1b** was synthesized using procedure outlined for **1a**. The compound was obtained as yellow viscous liquid (110 mg, 34%). FT-IR $\tilde{\nu}_{max}$ = 3373, 2926, 1744, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 7.28 (d, J = 8.5 Hz, 2H), 6.89 (d, J = 8.4 Hz, 2H), 5.52 (s, 1H), 4.10 (s, 2H), 3.25–3.22 (m, 4H), 2.16 (s, 3H), 1.92–1.91 (m, 6H), 1.52 (s, 8H), 0.90 ppm (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 190.9, 187.4, 171.4, 166.4, 152.0, 149.4, 142.9, 139.1, 138.6, 136.3, 129.9, 121.5, 47.6, 43.2, 38.4, 33.5, 28.9, 22.9, 14.3, 12.7, 12.1, 11.2 ppm; HRMS (ESI-TOF) for $[C_{25}H_{31}NO_5S+H]^+$: calcd 458.2002, found 458.2002.

Synthesis of methyl 2-hydroxy-5-((((4-((3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanoyl)oxy)benzyl)thio)carbonyl)amino)benzoate (NQO1-Mes): Compound NQO1-Mes was synthesized using procedure outlined for **1a**. DMF was used as the solvent for reaction. The compound was obtained as yellow solid (146 mg, 43% of 2 steps). FT-IR $\tilde{\nu}_{max}$ = 3330, 1745, 1681, 1643 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 10.63 (s, 1H), 7.91 (s, 1H), 7.48 (bs, 1H), 7.35 (dd, J = 8.9 Hz, 2.8 Hz, 1H), 7.29 (d, J = 8.6 Hz, 2H), 6.89 (d, J = 8.5 Hz, 3H), 4.13 (s, 2H), 3.90 (s, 3H), 3.23 (s, 2H), 2.15 (s, 3H), 1.91–1.90 (m, 6H), 1.51 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 190.8, 187.4, 171.5, 170.0, 158.6, 151.9, 149.5, 142.8, 139.2, 138.6, 135.7, 130.0, 121.6, 118.1, 112.1, 52.5, 47.6, 38.4, 33.7, 28.9, 14.4, 12.7, 12.1 ppm; HRMS (ESI-TOF) for $[C_{30}H_{31}NO_8S+H]^+$: calcd 566.1848, found 566.1848.

Methylene Blue method for H_2S detection:[6b] Each assay described here was done in triplicate in vials with closed lids, containing 746 μL of PBS, 8 μL of H_2S donor (5 mM stock in DMSO), 20 μL of NADH (10 mM stock), 8 μL of NQO1 (1 mg mL^{-1}), 8 μL carbonic anhydrase (1% stock in PBS buffer) and 10 μL $Zn(OAc)_2$ (40 mM stock in H_2O). The reaction mixture was incubated at 37 °C for 4 h. 100 μL aliquot was removed after pre-determined time points from each reaction vial and diluted with 100 μL of $FeCl_3$ (30 mM stock in 1.2 M HCl) and 100 μL of *N,N*-dimethyl-*p*-pheneylenediamine sulfate (20 mM stock in 7.2 M HCl). The methylene blue mixture was further incubated at 37 °C for 30 min. After completion of methylene blue complex formation, aliquots were transferred to a 96 well plate (250 μL/well) and the absorbance spectra were collected from 500 to 800 nm on a plate reader. A similar protocol was followed to monitor the generation of H_2S from NQO1-Mes. Each assay was done in triplicate in vials with closed lids, containing 374 μL of PBS, 4 μL of NQO1-Mes (10 mM stock in DMSO), 10 μL of NADH (10 mM stock), 4 μL of NQO1 (1 mg mL^{-1}), 4 μL carbonic anhydrase (1% stock in PBS buffer) and 4 μL $Zn(OAc)_2$ (40 mM stock in H_2O). Dicoumarol **8**, is a known inhibitor of NQO1. For the control with NQO1 inhibitor, 100 μM (10 mM stock in 0.1 N NaOH) of the compound **8** was added to the reaction mixture containing NQO1-Mes in the presence of NQO1, NADH and CA. After 4 h, 100 μL aliquot was removed from each reaction vial and diluted with 100 μL of $FeCl_3$ (30 mM stock in 1.2 M HCl) and 100 μL of *N,N*-dimethyl-*p*-pheneylenediamine sulfate (20 mM stock in 7.2 M HCl). The mixture was incubated at 37 °C for 30 min. After completion of methylene blue complex formation, aliquots were transferred to a 96 well plate (250 μL/ well) and the absorbance spectra were collected from 500 to 800 nm on a plate reader.

HPLC analysis: The decomposition of compound **1a** was followed by HPLC. A stock solution of H_2S donors (2.5 mM) was prepared in DMSO. The stock of NQO1 (1 mg mL^{-1}) and NADH (10 mM) were prepared in phosphate buffer (pH 7.4, 10 mM). The reaction mixture consisted of compound (25 μM) in buffer containing NQO1 (10 μg mL^{-1}) and NADH (100 μM) and incubated at 37 °C. At predetermined time points, an aliquot of the reaction mixture was removed, filtered (0.22 micron filter) and injected (50 μL) in a high performance liquid chromatography (HPLC Agilent Technologies 1260 Infinity). The mobile phase was H_2O/ACN. The stationary phase was C-18 reverse phased column (Phenomenex, 5 μm, 4.6× 250 mm). A multistep gradient was used with a flow rate of 1 mL min^{-1} starting with →0–13 min, 50:50 to 10:90→13–16 min, 10:90 to 10:90→16–20 min, 10:90 to 50:50.

A similar protocol was used for compound NQO1-Mes. The reaction mixture consisted of compound (50 μM) in buffer containing NQO1 (10 μg mL^{-1}) and NADH (100 μM) and incubated at 37 °C. At predetermined time points, an aliquot of the reaction mixture was removed, filtered (0.22 micron filter) and injected (50 μL) in a high performance liquid chromatography (HPLC Agilent Technologies 1260 Infinity). The mobile phase was H_2O/ACN. The stationary phase was C-18 reverse phased column (Phenomenex, 5 μm, 4.6× 250 mm). A multistep gradient was used with a flow rate of 1 mL min^{-1} starting with →0–5 min, 40:60 to 25:75→5–10 min, 25:75 to 10:90 → 10–15 min, 10:90 to 0:100→15–17 min 0:100 to 0:100→17–20 min, 0:100 to 40:60→20–22 min, 40: 60.

H_2S detection using sulfide selective electrode: Compound NQO1-Mes (10 μL, 5 mM) was added to PBS pH 7.4 (925 μL) containing CA (5 μL, 1% stock), NADH (50 μL, 10 mM) and NQO1 (10 μL, 1 mg mL^{-1}) at 37 °C in a closed vial. The H_2S produced was detected using 5 mm H_2S sensitive microelectrode (ISO-H2S-100) attached to a TBR 4100 Free Radical Analyser (WPI) and shown as picoamps current generated. A standard calibration curve was generated using a freshly prepared Na_2S solution in PBS buffer by following the manufacturer's protocol.

Cell Culture: Human breast carcinoma, MCF-7 cells and wild type mouse embryonic fibroblast, WT-MEF cells were were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (FBS), 1% Penicillin Streptomycin antibiotic. Human colon adenocarcinoma, DLD-1 cells, were grown in RPMI 1640 media containing 10% (v/v) fetal bovine serum (FBS), 1% Penicillin Streptomycin antibiotic.

Cellular persulfidation protocol: Human colon carcinoma, DLD-1 cells were seeded in a 12 well plate at a concentration of 0.1×10^6 cells/well in RPMI media supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic solution and incubated in an atmosphere of 5% CO_2 at 37 °C overnight. Next day, the cells were treated with test compounds (10 μM) and Na_2S (200 μM) for 1 h. The cells were washed twice with sterile PBS after the treatment was over. Cells were then fixed by incubating on ice cold methanol at −20 °C for 20 min and subsequently permeabilized using ice cold acetone −20 °C for 5 min. The cells were then washed with PBS and treated with 50 mM HEPES containing triton (1%) and MSBT (10 mM) for

overnight at room temperature. Cells were again washed with PBS (3 times) and further incubated with CN-BOT (25 μM) in PBS for 1 h at 37 °C. Finally, cells were washed 5 times with PBS and imaged using IX83 microscope (Olympus Inc., Japan). The data that we have presented is representative of 3 independent biological replicates. Each experiment >20 fields were imaged at identical image acquisition settings.

Cell Viability Assay. (a) MTT assay: The cytotoxicity effect of the H_2S donors was tested against various cell lines using a similar protocol. Human colon cancer cells, DLD-1, were seeded at a concentration of 1×10^4 cells/well overnight in a 96-well plate in complete RPMI media. Cells were exposed to varying concentrations of the test compounds prepared as a DMSO stock solution so that the final concentration of DMSO was 0.5%. The cells were incubated for 24 h at 37 °C. A solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was prepared using 3.5 mg in 7 mL RPMI media. 100 μL of the resulting solution was added to each well. After 4 h incubation, the media was removed carefully and 100 μL of DMSO was added to each well. Spectrophotometric analysis of each well using a microplate reader (Thermo Scientific Varioskan) at 570 nm was carried out to estimate cell viability. A similar protocol was followed for wild type mouse embryonic fibroblast (WT-MEF cells) and the media used was complete DMEM media. Human breast carcinoma, MCF-7 cells were seeded at a concentration of 1×10^3 cells/well in a 96 well plate overnight in complete DMEM media. The cells were treated with varying concentrations of compounds for 72 h following which the cell viability was measured using MTT.

Cell Viability Assay. (b) LDH Release Assay: Lactate Dehydrogenase (LDH) release was monitored to determine the cytotoxicity of the compounds, as a measurement of necrotic cell death using CCK036, EZCount™ LDH cell assay kit from Himedia Cell culture. The manufacturer's protocol was followed to determine the cytotoxicity. Briefly, human breast carcinoma, MCF-7 cells were seeded at a concentration of 1×10^4 cells/well in a 96 well plate and incubated for 16 h. After the cells were attached the media was removed and replaced with fresh media containing varying concentrations of the compound **1a** with maximum DMSO concentration of 0.5%. The cells were incubated for another 24 h at 37 °C. After 24 h cells control was treated with 10 μL of lysis buffer and incubated for 45 min. Next, the plate was centrifuged for 15 min at 1500 rpm to settle down the cell debris. Following this, 50 μL of the media was transferred to another 96 well plate to which 50 μL of LDH reagent was added. The plate was incubated in Varioskan microtiter plate reader at 37 °C and the absorbance was measured after 25 min at 490 nm. The absorbance was plotted as a measure of cell viability with respect to the DMSO control.[28]

Protection against oxidative stress: Human colon adenocarcinoma cells DLD-1 were seeded in a 96-well plate with 10^4 cells/well in RPMI media supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic solution and incubated in an atmosphere of 5% CO_2 at 37 °C for 16 h. Stock solutions of compounds were prepared in RPMI with final concentration of DMSO not exceeding 0.5%. After 16 h, the cells were co-treated with different concentrations of the compound and JCHD (15 μM). The cells were incubated for 24 h at 37 °C. The media was removed after 24 h and the cells were treated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) at a concentration of 0.5 mg mL^{-1}. A stock solution of MTT was prepared by dissolving 3.5 mg in 7 mL RPMI media and 100 mL of this stock was added to each well. After incubating at 37 °C for 4 h, the media was carefully removed and 100 μL of DMSO was added to each well. Absorbance at 570 nm was recorded using a microplate reader (Thermo Scientific Varioskan) to estimate the cell viability. A similar protocol was followed for demonstrating the effects of H_2S donors against JCHD induced oxidative stress in WT-MEF cells. Cells were grown in DMEM media supplemented with 10% FBS and 1% antibiotic solution.

Acknowledgements

The Council for Scientific and Industrial Research (CSIR) is acknowledged for research fellowships. The authors would also like to thank Infosys Foundation for international travel grant.

Conflict of interest

The authors declare no conflict of interest.

Keywords: carbonyl sulfide · hydrogen sulfide · persulfidation · prodrug

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Manuscript received: August 17, 2019
Revised manuscript received: September 7, 2019
Accepted manuscript online: September 10, 2019
Version of record online: ■■ ▮▮, 0000

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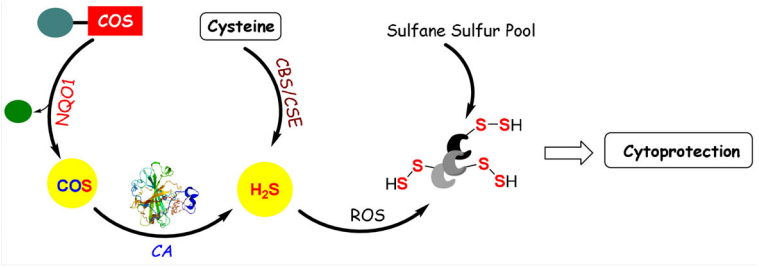

NQO1 triggered release of COS induces protein persulfidation within cells and likely contributes to cytoprotective effects.

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Carbonyl Sulfide (COS) Donor Induced Protein Persulfidation Protects against Oxidative Stress