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Hydropersulfides Inhibit Lipid Peroxidation and Protect Cells from Ferroptosis

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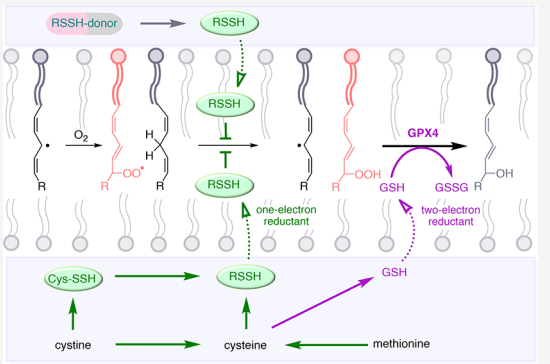
ABSTRACT: Hydropersulfides (RSSH) are believed to serve important roles *in vivo*, including as scavengers of damaging oxidants and electrophiles. The α-effect makes RSSH not only much better nucleophiles than thiols (RSH), but also much more potent H-atom transfer agents. Since HAT is the mechanism of action of the most potent small-molecule inhibitors of phospholipid peroxidation and associated ferroptotic cell death, we have investigated their reactivity in this context. Using the fluorescence-enabled inhibited autoxidation (FENIX) approach, we have found RSSH to be highly reactive toward phospholipid-derived peroxyl radicals (k_{inh} = 2 × 10^5 M^{-1} s^{-1}), equaling the most potent ferroptosis inhibitors identified to date. Related (poly)sulfide products resulting from the rapid self-reaction of RSSH under physiological conditions (e.g., disulfide, trisulfide, H_2S) are essentially unreactive, but combinations from which RSSH can be produced *in situ* (i.e., polysulfides with H_2S or thiols with H_2S_2) are effective. *In situ* generation of RSSH from designed precursors which release RSSH via intramolecular substitution or hydrolysis improve the radical-trapping efficiency of RSSH by minimizing deleterious self-reactions. A brief survey of structure−reactivity relationships enabled the design of new precursors that are more efficient. The reactivity of RSSH and their precursors translates from (phospho)lipid bilayers to cell culture (mouse embryonic fibroblasts), where they were found to inhibit ferroptosis induced by inactivation of glutathione peroxidase-4 (GPX4) or deletion of the gene encoding it. These results suggest that RSSH and the pathways responsible for their biosynthesis may act as a ferroptosis suppression system alongside the recently discovered FSP1/ubiquinone and GCH1/BH_4/DHFR systems.



INTRODUCTION

Sulfhydration of protein cysteine thiols has emerged as a ubiquitous physiologic post-translation modification.[1−5] Reactions of the resultant hydropersulfides (RSSH)—and the polysulfides (RSS_nR) derived therefrom—are widely believed to exert the biological effects attributed to H_2S.[6−9] Numerous reports suggest that RSSH derived from cysteine and glutathione are omnipresent in mammalian cells, tissue, and plasma, prompting intensive investigations of their role.[10,11] Given the substantially enhanced nucleophilicity of RSSH relative to thiols (RSH),[12,13] they are often invoked as intermediates in cellular signaling pathways that respond to electrophile and/or oxidant stress[3,14−19] (Figure 1A, top). Importantly, while protein thiol modification/oxidation is generally irreversible, protein hydropersulfides can be reduced to restore protein thiols once the stress is resolved.[4,5,20,21] Alternatively—or in addition to a signaling function—sulfhydration may have evolved as a direct protective mechanism against electrophilic/oxidative stress. In this context, one-electron reactivity should also be considered (Figure 1A, bottom), given the weak RSS−H bond (70 kcal/mol) and low oxidation potential of the persulfide anion, both properties resulting from the high stability of the perthiyl radical.[22,23]

Radical reactions are most destructive in the lipid domains of the cell, where an efficient chain reaction leads to the accumulation of lipid hydroperoxides.[24] This radical chain reaction, known as autoxidation, is generally propagated by H-atom abstraction from a polyunsaturated lipid by a lipid-derived peroxyl radical. This yields a lipid hydroperoxide and a lipid-derived alkyl radical, which is subsequently oxygenated to afford another lipid-derived peroxyl radical (Figure 1A, bottom).[25] Fragmentation of the resultant (phospho)lipid hydroperoxides leads to loss of membrane integrity and eventual cell death now known as ferroptosis.[26−28] Ferroptosis is suppressed primarily by glutathione peroxidase-4 (GPX4), which catalyzes the reduction of (phospho)lipid hydro-

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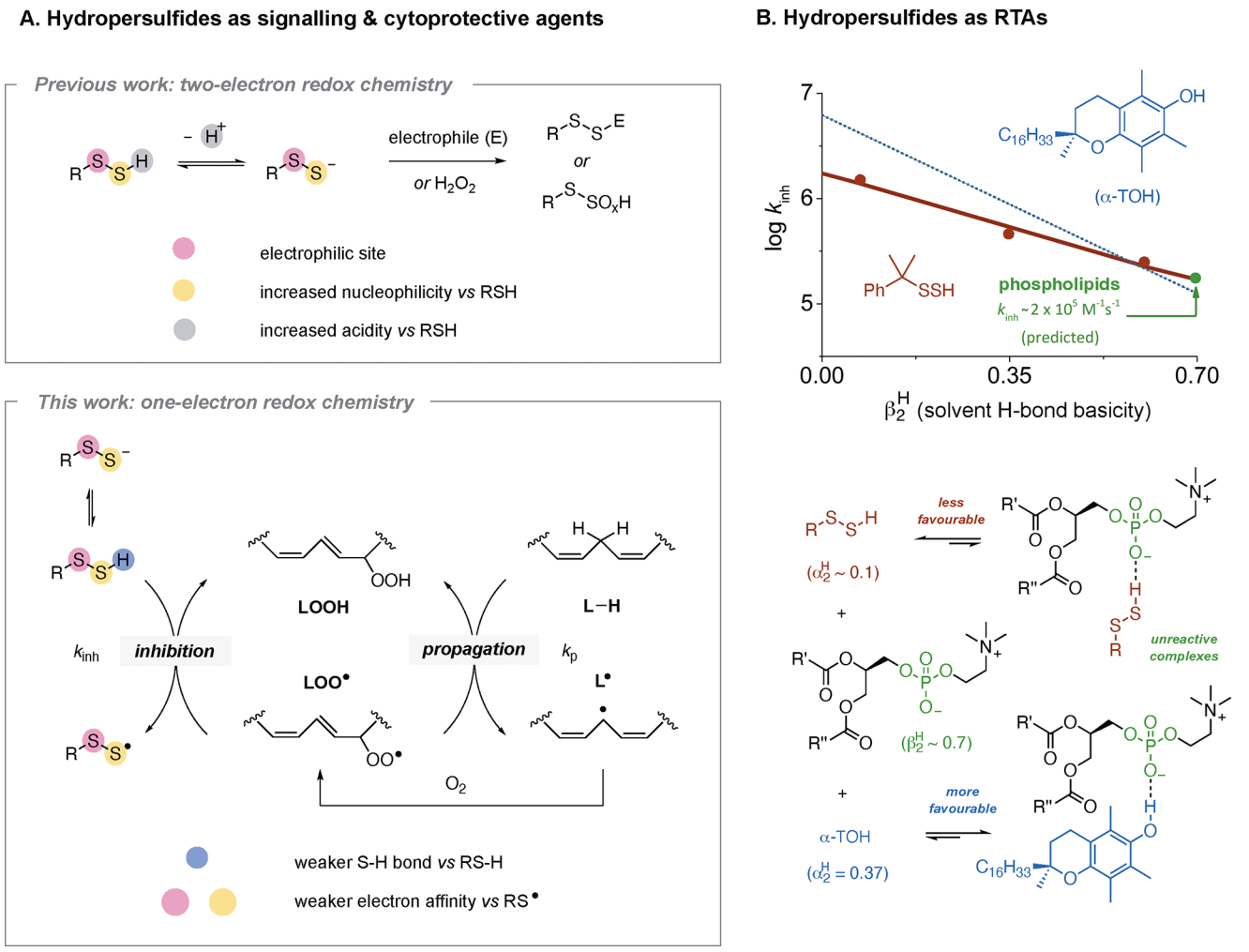

Figure 1. (A) RSSH are generally invoked as two-electron reductants and ascribed protective roles associated with their ability to react with oxidants and electrophiles. In this work, the ability of RSSH to protect cells by inhibition of (phospho)lipid peroxidation and associated cell death (ferroptosis) is investigated. (B) Correlations of inhibition rate constants for cumyl-SSH and α-TOH with solvent H-bond basicity (β_2^H)[22] suggest that RSSH will be more potent than α-TOH in phospholipids, which are strong H-bond acceptors. RSSH are much weaker H-bond donors than α-TOH, meaning more "free" (non-H-bonded) RTA to inhibit lipid peroxidation.

peroxides to their corresponding (innocuous) alcohols by glutathione.[29] Lipophilic radical-trapping antioxidants (RTAs),[30−34] such as α-tocopherol (α-TOH)[35]—the most biologically active form of vitamin E—also contribute to ferroptosis suppression.[28,36] RTAs generally transfer a H-atom to a chain-propagating lipidperoxyl radical, yielding a hydroperoxide and an RTA-derived radical that does not carry on the chain reaction. RTAs can be acquired from the diet (e.g., α-TOH) or synthesized on demand (e.g., tetrahydrobiopterin[37,38]). RTAs can also be regenerated from their oxidation products: α-TOH and coenzyme Q_{10} by ferroptosis suppressor protein-1[39,40] and tetrahydrobiopterin by dihydrofolate reductase,[37,38] both at the expense of the ubiquitous cellular reductant NAD(P)H.

A short time ago, we found that RSSH were among the best H-atom donors ever identified, reacting with a variety of radicals with rate constants several orders of magnitude greater than those measured for RSH.[22] Among the various types of H-atom transfer (HAT) reactions we studied were those with peroxyl radicals, for which rate constants of 1 to 2 × 10^6 M^{-1} s^{-1} were determined, similar to those measured for α-TOH under the same conditions. However, the rates of the reactions of RSSH were largely invariant with changes in the medium, whereas those for almost all good RTAs, including α-TOH, are highly medium-dependent.[36,41,42] For example, the rate constant for HAT from α-TOH to peroxyl radicals drops roughly 100-fold on going from chlorobenzene to phospholipid bilayers (Figure 1B, top). This rate reduction is believed to arise due to H-bond formation between α-TOH and the phosphodiester headgroup (Figure 1B, bottom), which lowers the concentration of "free" (i.e., reactive) α-TOH. Importantly, we found that RSSH are very poor H-bond donors,[22] suggesting that they should be even more potent than α-TOH as inhibitors of phospholipid peroxidation and, further, that they may serve similarly to suppress ferroptotic cell death. Herein we present the details of our investigation of this possibility, directly with persistent RSSH and indirectly with RSSH precursors and combinations of H_2S and H_2S_2 with polysulfides and thiols, respectively.

RESULTS

I. Hydropersulfides Are Potent Inhibitors of Phospholipid Peroxidation. The reactivity of select RSSH as inhibitors of (phospho)lipid peroxidation was studied using

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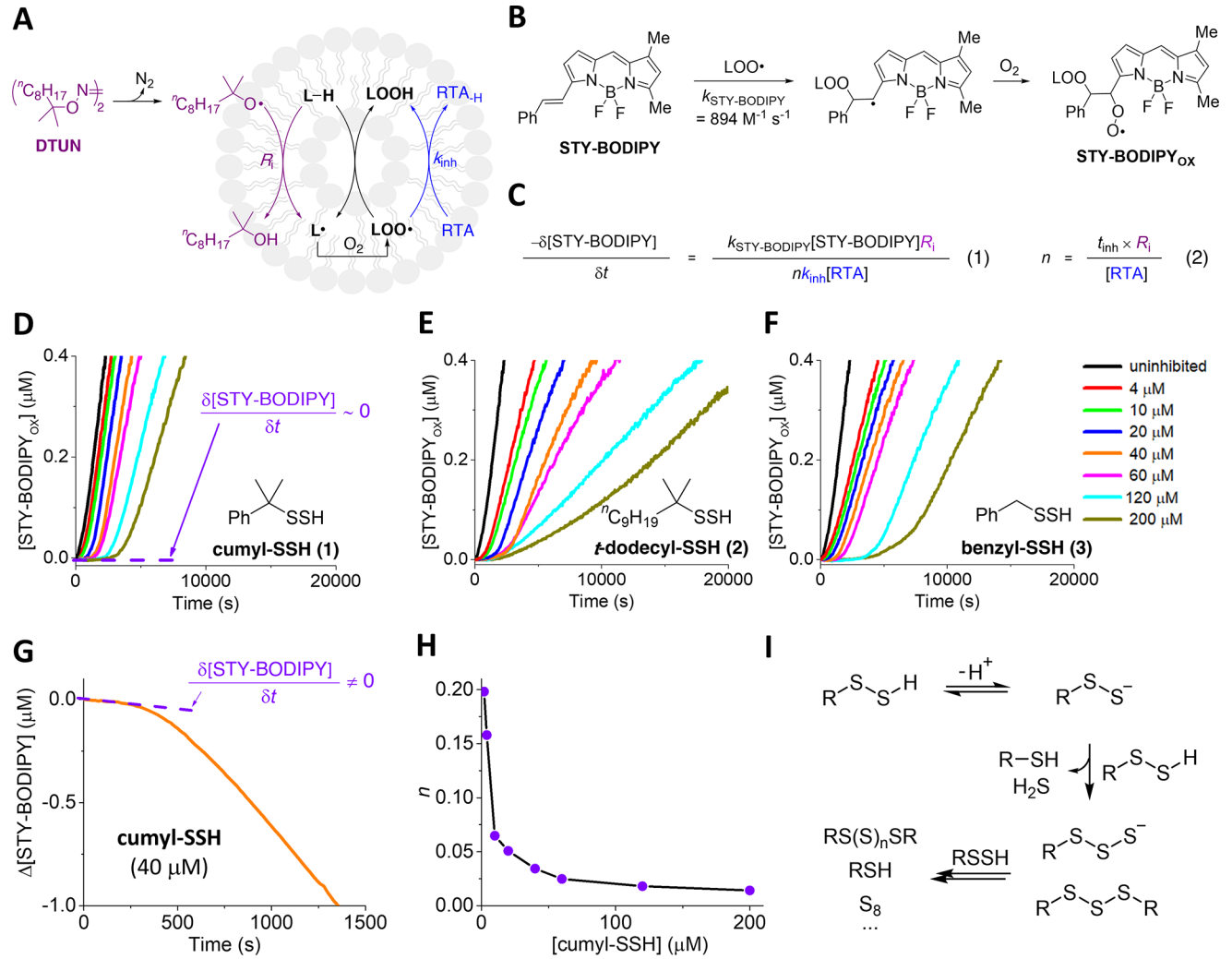

Figure 2. (A) DTUN decomposes to form lipophilic alkoxyl radicals at a constant rate R_i to initiate lipid peroxidation in liposomal egg phosphatidylcholine (1 mM, 100 nm particles) in phosphate-buffered saline (PBS) at pH 7.4. (B) STY-BODIPY is used as the signal carrier. (C) The inhibition rate constants (k_{inh}) and radical-trapping stoichiometries (n) of added RTAs (i.e., RSSH) can be derived from the rates of STY-BODIPY oxidation and the length of the inhibited periods (t_{inh}), respectively. (D–F) Representative co-autoxidations of STY-BODIPY (1 μM) and PC liposomes (1 mM) in PBS (10 mM) at pH 7.4 initiated by DTUN (200 μM) at 37 °C and inhibited by cumyl-SSH, *t*-dodecyl-SSH, and benzyl-SSH (4–200 μM). Reaction progress was monitored by fluorescence (λ_{ex} = 488 nm, λ_{em} = 518 nm). (G) Representative co-autoxidation inhibited by cumyl-SSH (40 μM) utilizing higher [STY-BODIPY] (10 μM) and monitored by absorbance. (H) Relationship between radical-trapping stoichiometry and hydropersulfide concentration illustrated for cumyl-SSH. (I) The known self-reaction of RSSH yields multiple products, including polysulfides, thiols, and H_2S.

the FENIX (fluorescence-enabled inhibited autoxidation) approach.[43] Therein, STY-BODIPY is co-autoxidized along with the polyunsaturated side chains of liposomal phospholipids (egg phosphatidylcholine). Reaction progress is monitored indirectly by accumulation of the fluorescent oxidation product (λ_{ex} = 488 nm, λ_{em} = 518 nm) as chain-propagating peroxyl radicals add to the styryl moiety of the probe (Figure 2B). Since the kinetics of STY-BODIPY oxidation are known under these conditions ($k_{STY\text{-}BODIPY}$ = 894 M^{-1} s^{-1}) and the rate of initiation from decomposition of the lipophilic di-*tert*-undecyl hyponitrite DTUN (R_i) is easily determined (Figure 2A, see Supporting Information), the inhibition rate constant (k_{inh}) and the radical-trapping stoichiometry (n) of an added RTA can be determined from the initial rate of the inhibited autoxidation and the length of the inhibited period (t_{inh}), respectively (Figure 2C). Representative results obtained from co-autoxidations inhibited by added cumyl-SSH (**1**), *t*-dodecyl-SSH (**2**), and benzyl-SSH (**3**), synthesized as described previously,[22,44] are shown in Figure 2D–F.

The RSSH derivatives were found to be highly reactive toward (phospho)lipid-derived peroxyl radicals, fully suppressing STY-BODIPY consumption under standard conditions, similarly to phenoxazine, the most reactive inhibitor identified to date.[30,32] To obtain nonzero rates of STY-BODIPY oxidation necessary for determination of k_{inh}, the concentration of STY-BODIPY had to be increased 10-fold and the reaction monitored by absorbance in lieu of fluorescence (Figure 2G).[43,45] Under these conditions, a well-defined inhibited period of reproducible rate was obtained, which yielded k_{inh} = (2.3 ± 0.3) × 10^5 M^{-1} s^{-1} for cumyl-SSH, in excellent agreement with the prediction based upon its RTA kinetics in organic solvents and H-bond acidity (cf. Figure 1B, top). However, despite this very high reactivity, the RSSH derivatives were characterized by very low radical-trapping stoichiometries (i.e., n < 0.20). Moreover, there was a marked

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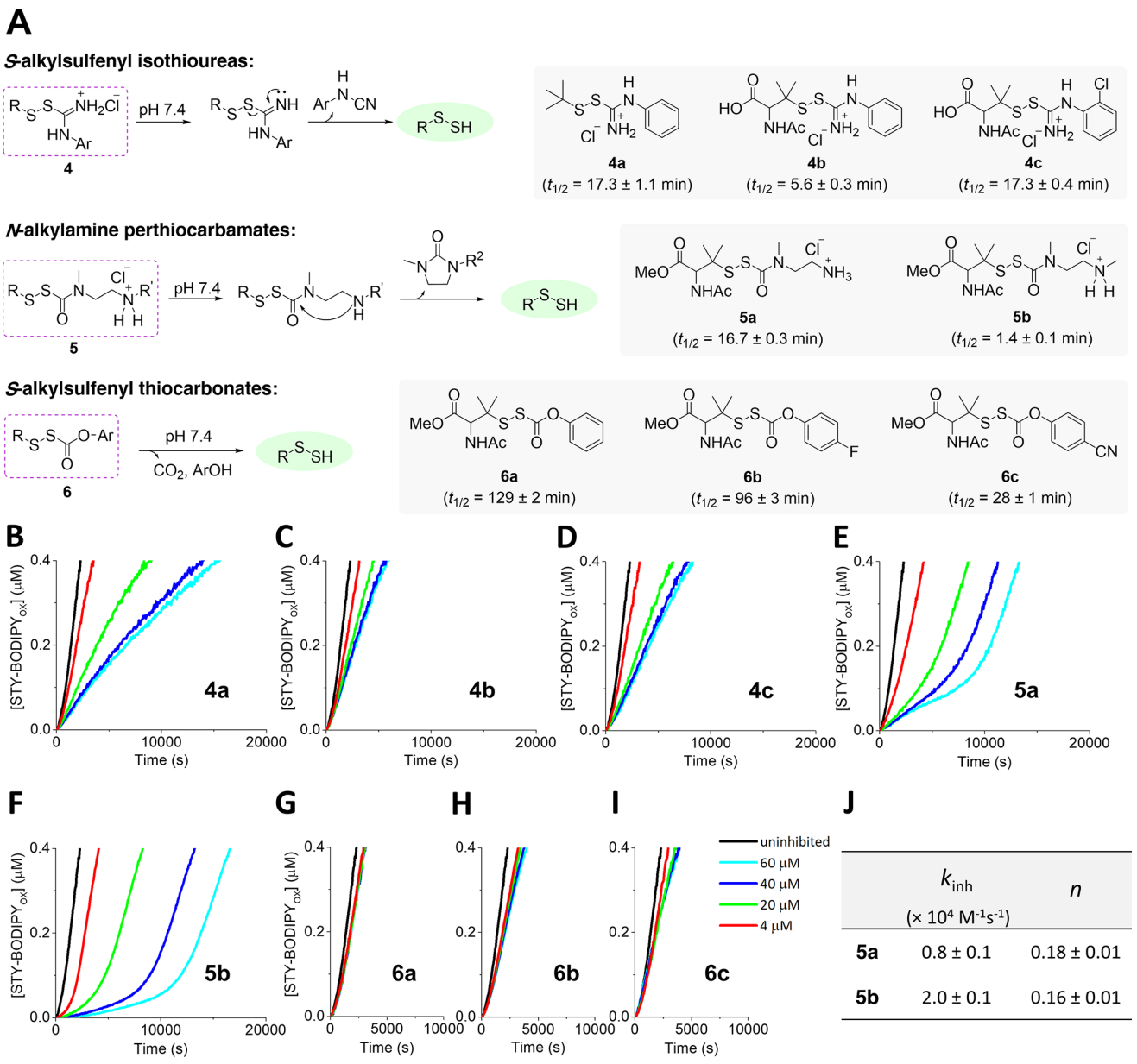

	k_{inh} (× 10^4 $M^{-1}s^{-1}$)	n
5a	0.8 ± 0.1	0.18 ± 0.01
5b	2.0 ± 0.1	0.16 ± 0.01

Figure 3. (A) RSSH precursors used in this work along with their respective half-lives measured in PBS (pH 7.4) containing DTPA at 37 °C (see refs 46−48). (B−I) Representative co-autoxidations of STY-BODIPY (1 μM) and PC liposomes (1 mM) in PBS (10 mM) at pH 7.4 initiated by DTUN (200 μM) at 37 °C and inhibited by RSSH precursors **4a−c**, **5a**, **5b**, and **6a−c**. Reaction progress was monitored by fluorescence (λ_{ex} = 488 nm, λ_{em} = 518 nm). (J) Inhibition rate constants and radical-trapping stoichiometries for precursors **5a** and **5b** (calculated with 20 μM data).

decrease in stoichiometry with increasing RSSH concentration (Figure 2H). The apparent second-order relationship between n and [RSSH] is consistent with the depletion of RSSH by its bimolecular self-reaction, which yields polysulfides, thiols, and H_2S (Figure 2I),[12,44,46] each of which are largely devoid of RTA activity (*vide infra*).

II. *In Situ* Formation Increases the Efficiency of Hydropersulfides as Inhibitors of Phospholipid Peroxidation. Given the instability of RSSH in aqueous solution, we considered whether *in situ* formation could improve RTA efficiency.[17,19,47] The Toscano group has developed RSSH precursors characterized by various release rates under physiological conditions; deprotonation at pH 7.4 initiates RSSH formation by elimination from *S*-alkylsulfenyl isothioureas (**4**)[48] and intramolecular substitution on *N*-alkylamine perthiocarbamates (**5**),[49] while *S*-alkylsulfenyl thiocarbonates (**6**)[50] undergo hydrolysis to afford RSSH (Figure 3A). We selected examples of each of these precursors to probe the impact of both half-life and polarity on their ability to suppress (phospho)lipid peroxidation. Representative results for inhibited co-autoxidations are shown in Figure 3B−I.

While the *S*-alkylsulfenyl thiosiothioureas **4a**, **4b**, and **4c** were only modest inhibitors at best and the *S*-alkylsulfenyl thiocarbonates **6a**, **6b**, and **6c** were essentially devoid of activity, the *N*-alkylamine perthiocarbamates **5a** and **5b** showed good dose-dependent RTA activity. The well-defined inhibited periods for **5a** and **5b** correspond to a radical-trapping stoichiometry of n = 0.18 and 0.16 (Figure 3J), much higher than when equivalent concentrations of RSSH were used directly (n < 0.05 when >20 μM). From the initial rates of the inhibited autoxidations, it is clear that the RTA activity of the faster-releasing **5b** ($t_{1/2}$ = 1.4 min) is superior to that of the

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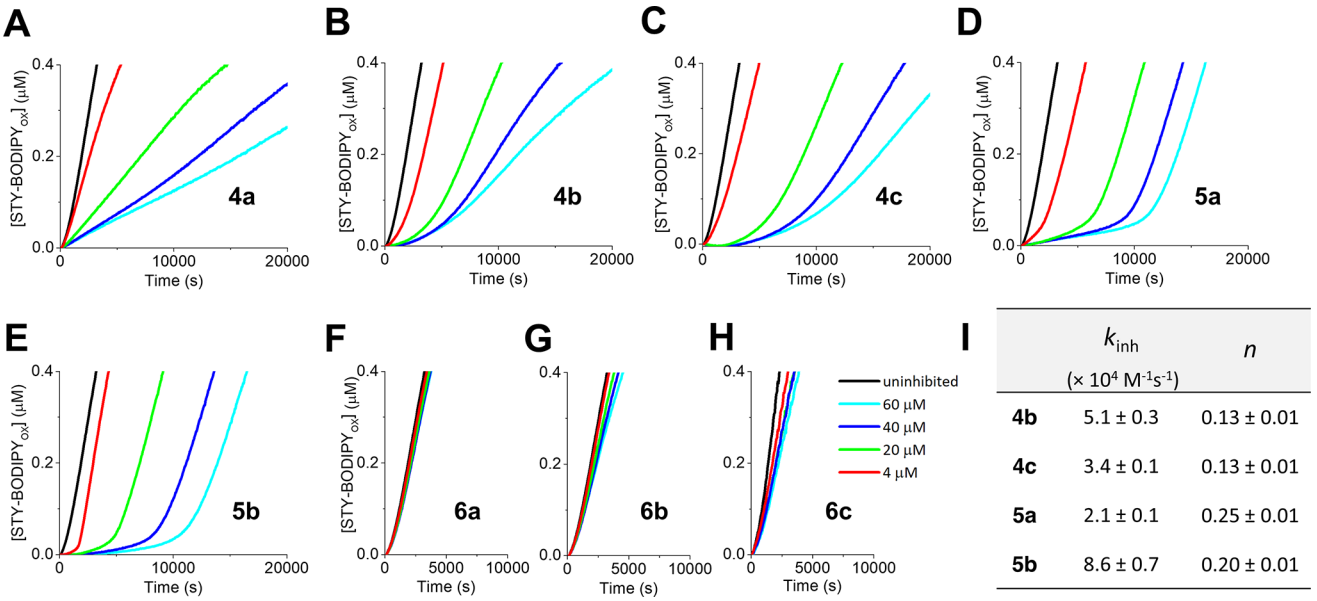

	k_{inh} (× 10^4 $M^{-1}s^{-1}$)	n
4b	5.1 ± 0.3	0.13 ± 0.01
4c	3.4 ± 0.1	0.13 ± 0.01
5a	2.1 ± 0.1	0.25 ± 0.01
5b	8.6 ± 0.7	0.20 ± 0.01

Figure 4. (A−H) Representative co-autoxidations of STY-BODIPY (1 μM) and PC liposomes (1 mM) in PBS (10 mM) at pH 7.4 initiated by MeOAMVN (200 μM) at 37 °C and inhibited by RSSH precursors **4a−c**, **5a**, **5b**, and **6a−c**. Reaction progress was monitored by fluorescence (λ_{ex} = 488 nm, λ_{em} = 518 nm). (I) Inhibition rate constants and radical-trapping stoichiometries for precursors **4b,c** and **5a,b** (calculated with 20 μM).

slower-releasing **5a** ($t_{1/2}$ = 16.7 min). Since the same RSSH is formed from each of **5a** and **5b**, it appears that although the lower release rate can minimize deleterious (self-) reactions, it also affords less RSSH to compete with propagation of (phosho)lipid peroxidation. This appears to be reinforced by results obtained with the *S*-alkylsulfenyl thiocarbonates **6a** ($t_{1/2}$ = 129 min), **6b** ($t_{1/2}$ = 96 min), and **6c** ($t_{1/2}$ = 28 min), which perhaps release RSSH too slowly to inhibit (phospho)lipid peroxidation under these conditions (more on this later). Since these precursors are less polar than the perthiocarbamates, we considered that RSSH formation may be stymied by slower hydrolysis due to partitioning to the lipid phase of the liposomes, but found similar kinetics for RSSH release in liposome suspensions (see Supporting Information). Interestingly, the less precipitous drop in n and k_{inh} as a function of [**5a**] or [**5b**] implies that RSSH already associated with the lipids undergoes deleterious self-reaction more slowly (see Supporting Information), consistent with the increased stability of RSSH in aprotic organic media.[22]

Since the RTA activities of **4a** and **4c** were significantly different from that of **5a** despite practically indistinguishable half-lives ($t_{1/2}$ = 17.3 vs 16.7 min, respectively), we wondered if poor partitioning of the RSSH derived from **4a**/**4c** to the lipid phase of the liposomes may limit their efficacy (and, by extension, the more rapidly releasing **4b**). To investigate this possibility, we replaced the highly lipophilic initiator DTUN with the more amphiphilic azo initiator MeOAMVN (2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) in our experiments. We have previously demonstrated that MeOAMVN initiates at the interfacial region of PC liposomes upon addition to the suspension, such that amphiphilic RTAs can react directly with MeOAMVN-derived radicals to suppress initiation of phospholipid peroxidation.[30,51,52] In the event, significantly enhanced inhibition was observed for **4a−c** in the MeOAMVN-initiated autoxidations (compare Figure 4A−C to Figure 3B−D), particularly for **4b** and **4c**, which now yield well-defined inhibited periods corresponding to n = 0.13 (Figure 4I). Still, the slow-releasing **6a**, **6b**, and **6c** were poor inhibitors (Figure 4F−H). Switching MeOAMVN for the water-soluble azo initiator AAPH (2,2-azobis(2-amidinopropane)dihydrochloride) further reinforces the notion that the carboxylate-bearing RSSH derived from **4b** and **4c** partition preferentially to the aqueous phase;[31,43] while the profiles of the other precursors are hardly different from those in the MeOAMVN-initiated autoxidations, those from **4b** and **4c** are further enhanced, with inhibited oxidation rates and inhibition periods now similar to those observed for **5a** and **5b** (see Supporting Information).

III. Hydropersulfides Are the Most Effective Inhibitors of Phospholipid Peroxidation among Sulfane Sulfur Species. Since RSSH are known to decompose rapidly in aqueous solution to form H_2S and trisulfide (at least, initially),[44] we carried out inhibited autoxidations in the presence of these compounds in order to confidently ascribe the observed RTA activity to RSSH. Only slight retardation of the oxidation was observed when the buffer was supplemented with up to 500 μM Na_2S (see Figure 5A and additional data in the Supporting Information), a concentration well in excess (10×) of any of the precursors and 2.5-fold greater than any of the authentic RSSH tested under otherwise identical conditions. Disulfides (cumyl and glutathione) had no substantial impact on the rate, but cumyl tri- and tetrasulfide were able to slightly retard the rate of autoxidation, but again, only ever so slightly so and at much higher concentrations (see Figure 5A and additional data in the Supporting Information). Of the trisulfide and tetrasulfide, the latter was slightly more reactive, consistent with our recent finding that tetrasulfides undergo efficient substitution by peroxyl radicals at elevated temperatures.[53] Trisulfides are significantly less reactive in this regard.[52] Reducing the concentration of initiator—first from 200 to 100 μM and then from 100 to 50 μM—to slow the rate of oxidation, yielded data that further reinforce that the trisulfide can inhibit the autoxidation, but that the reactivity is nonetheless relatively poor (see Supporting Information). Overall, it is clear that the RTA activity observed for RSSH

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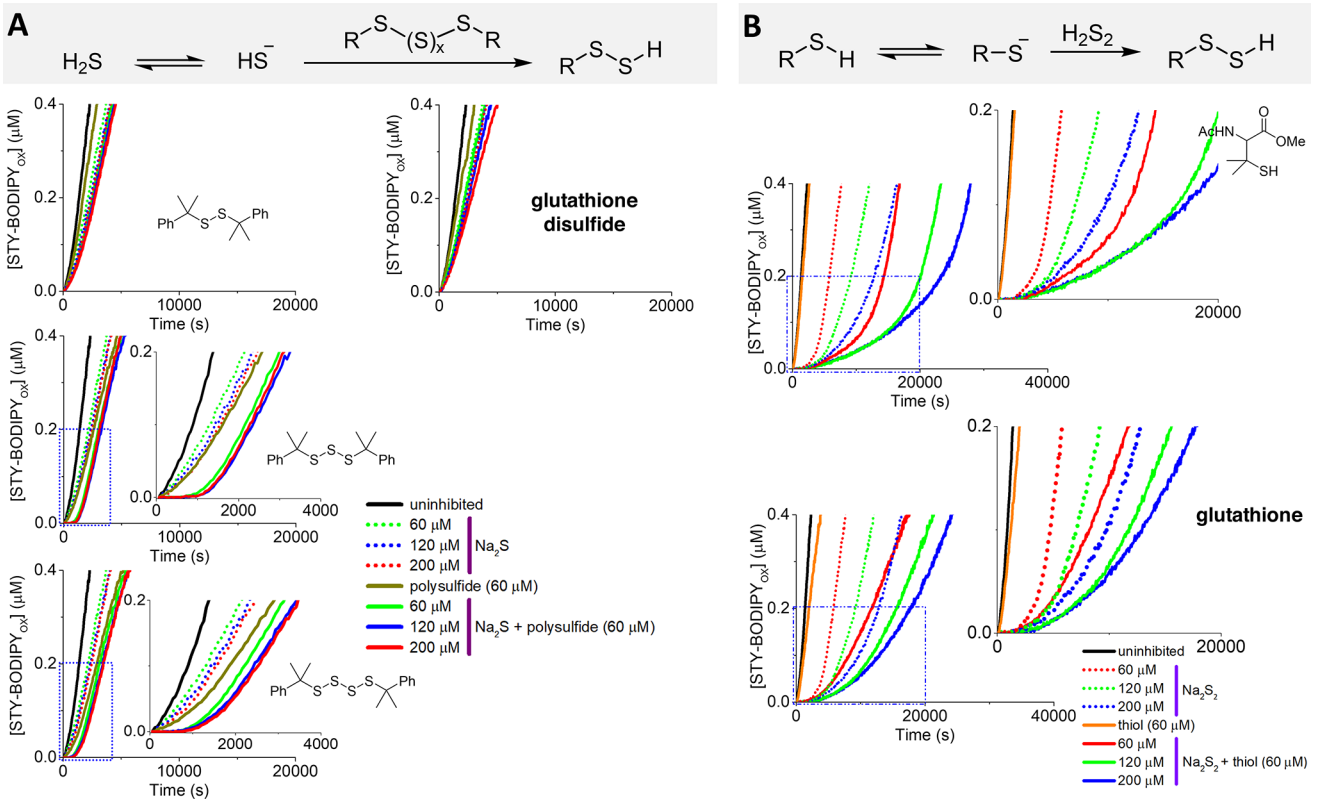

Figure 5. Representative co-autoxidations of STY-BODIPY (1 μM) and PC liposomes (1 mM) in PBS (10 mM) at pH 7.4 initiated by DTUN (200 μM) at 37 °C and inhibited by (A) H_2S (formed from Na_2S, 60−200 μM), cumyl polysulfides (60 μM), or the combination thereof; (B) H_2S_2 (formed from Na_2S_2, 60−200 μM), thiols (60 μM), or the combination thereof. Reaction progress was monitored by fluorescence (λ_{ex} = 488 nm, λ_{em} = 518 nm).

and/or their precursors is, in fact, due to RSSH and not decomposition products thereof.[54]

Although the marginal activities of the polysulfides were insufficient to underlie the RTA activity of either the authentic RSSH or the more reactive of the RSSH precursors, they were similar to precursors **6a**, **6b**, and **6c**. Slowing the rate of oxidation to enable competition of **6a** and **6c** with STY-BODIPY for chain-carrying peroxyl radicals (again, by lowering the initiator concentration) makes the similar reactivity of **6a**/**6c** and the trisulfides even more evident (see Supporting Information). Interestingly, in the initial characterization of **6a**−**c**, RSSH yields appeared to be limited by reactions of the released RSSH with the precursors themselves, which lead to trisulfides.[50] Thus, given the fact that RSSH derived from precursors **6** are the same as those derived from precursors **5** and that the rate of RSSH formation from at least one of **6** (i.e., **6c**) is similar to that of at least one of **5** (i.e., **5a**), it seems reasonable to suggest that the poor performance of precursors **6** as inhibitors of lipid peroxidation derives from the high reactivity of the initially released RSSH with the precursors themselves. Consistent with this suggestion, addition of either **6a** or **6c** to autoxidations inhibited by **5a** was detrimental (see Supporting Information). The fact that precursors **6** are uncharged and expected to concentrate in the lipid phase presumably facilitates this problematic side-reaction.

In addition to their biosynthesis, RSSH can be formed spontaneously from reactive sulfur species, e.g., the reaction of H_2S with oxidized thiols such as disulfides and sulfenic acids (Figure 5A).[55] While the combination of H_2S (derived from Na_2S) and disulfides (cumyldisulfide, glutathione disulfide, or *iso*-propyldisulfide; see Supporting Information) did not lead to suppression in the rate of lipid peroxidation, the addition of H_2S to autoxidations retarded by the trisulfide and tetrasulfide was clearly of benefit. In fact, these autoxidations were fully suppressed similarly to what was observed with authentic RSSH. Although the product of RSSH RTA activity is a perthiyl radical (RSS$^{\bullet}$) that can combine with another to reform tetrasulfide, additional H_2S did not substantially prolong the inhibited period, presumably due to the same competing reactions that erode the efficiency of RSSH as an RTA when it is used directly (*cf.*Figure 2I). Since RSSH can also be produced from metathesis with thiols and sulfane sulfides, polysulfides, and inorganic polysulfides (Figure 5B),[56] we also investigated representative examples of these combinations. Indeed, while acetyl-penicillamine, its methyl ester, and glutathione were devoid of RTA activity, they substantially improved the RTA activity of hydrogen persulfide (generated from Na_2S_2, Figure 5B and Supporting Information). Of the three thiols tested, the methyl ester of acetyl-penicillamine displayed the best synergy with H_2S_2, presumably since the resultant RSSH is the most lipophilic of the three (Figure 5B and Supporting Information). A comparison of the co-autoxidation traces in the presence of only H_2S (Na_2S) and H_2S_2 (Na_2S_2) underscores the benefit of a second sulfur atom to RTA activity.

IV. Hydropersulfides Inhibit Ferroptosis. To determine whether the RTA activity characterized above translates to cells, we examined whether RSSH and their precursors could inhibit ferroptotic cell death. Ferroptosis was induced in Pfa1

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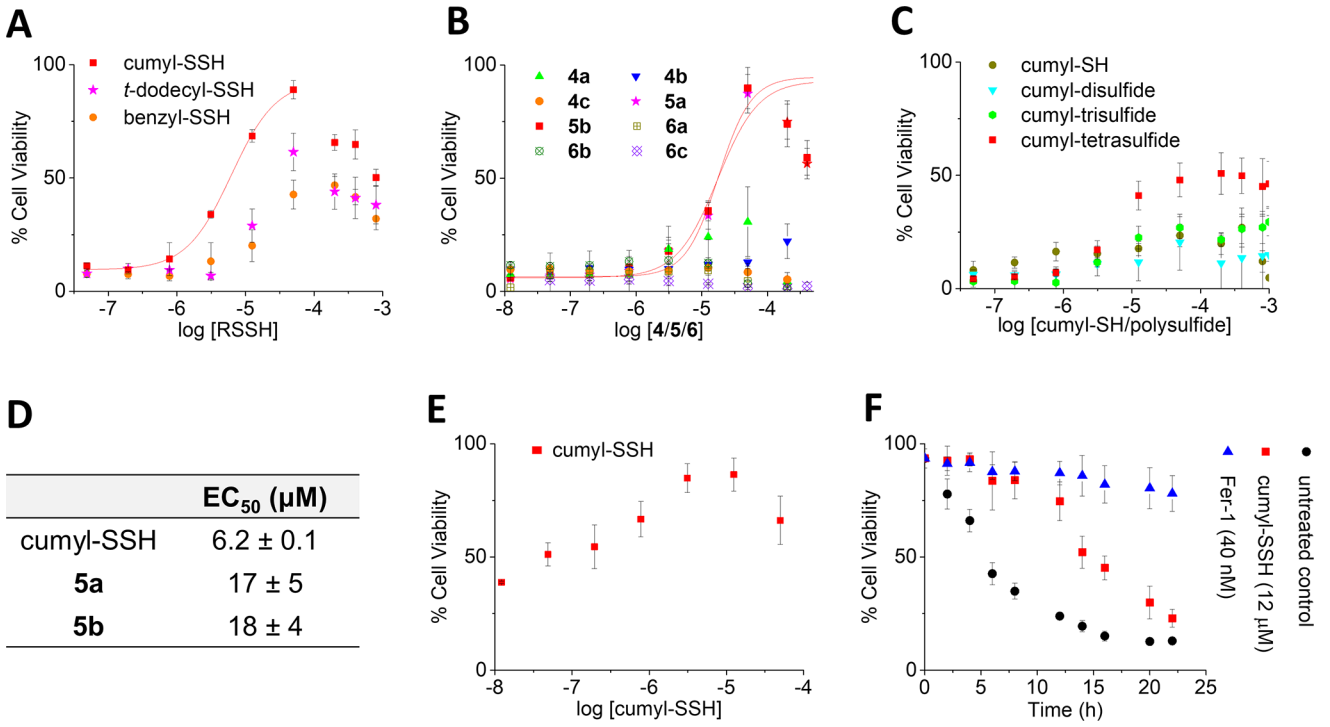

	EC_{50} (μM)
cumyl-SSH	6.2 ± 0.1
5a	17 ± 5
5b	18 ± 4

Figure 6. Rescue of RSL3-induced ferroptosis in Pfa1 MEFs by (A) authentic RSSH, (B) RSSH precursors, and (C) cumyl sulfur species with (D) corresponding EC_{50} values. (E) Rescue of tamoxifen-induced ferroptosis in Pfa1 MEFs following a 24 h preincubation with tamoxifen (700 nM) by cumyl-SSH (6 h). (F) Cell viability following 24 h tamoxifen treatment of Pfa1 MEFs in the presence of cumyl-SSH (12 μM) and Fer-1 (40 nM) as a function of time compared to untreated (vehicle-only) cells.

mouse embryonic fibroblasts (MEFs)[57] with (1*S*,3*R*)-RSL3,[58] the prototype GPX4 inhibitor, and cell viability was determined by the AquaBluer assay. Representative results are shown in Figure 6. Of the three authentic RSSH (Figure 6A), only cumyl-SSH appeared to fully rescue cells (EC_{50} = 6.2 μM). Beyond 50 μM, cell viability began to decrease, indicating cytotoxicity that was even more apparent for the *t*-dodecyl and benzyl hydropersulfides, which could only partially rescue cells from ferroptosis (see Supporting Information for corresponding cytotoxicity data in untreated cells). The RSSH precursors were also evaluated (Figure 6B), with the perthiocarbamates **5a** and **5b** (EC_{50} ~ 18 μM) proving most effective, fully consistent with their clear superiority in the FENIX studies. Also consistent with the foregoing results in liposomes, the related thiol, disulfide, trisulfide, and tetrasulfide species resulting from cumyl-SSH decomposition were unable to rescue cells from ferroptosis (Figure 6C).

Since RSSH are known to be excellent nucleophiles,[55] and RSL3 is a good electrophile that inhibits GPX4 by covalent attachment to its active site selenocysteine,[58,59] it was plausible that RSSH suppressed cell death in the foregoing experiments by reacting directly with RSL3. To probe this possibility, we also carried out a set of experiments wherein ferroptosis was induced by genetic deletion of *gpx4*.[57] The Pfa1 MEFs feature *gpx4* genes flanked by *loxP* sites, which direct deletion of the gene by Cre recombinase upon tamoxifen treatment. Given their relatively short lifetime, rather than incubating RSSH with the cells for 24 h and then assessing cell viability (as is generally done),[57] they were assessed at 6 h, similarly to the RSL3 experiment. Under these conditions, cumyl-SSH rescued cells in a dose-dependent manner[60] (Figure 6E). Furthermore, we assessed cell viability as a function of time following 24 h incubation of the Pfa1 cells with tamoxifen and addition of either the archetype inhibitor Fer-1 or cumyl-SSH. As shown in Figure 6F, both cumyl-SSH and Fer-1 prolonged cell viability, relative to the untreated control. Cumyl-SSH was found to be inferior to Fer-1, which was expected on the basis of its intrinsic instability. Nevertheless, these results demonstrate that RSSH are *bona fide* ferroptosis suppressors.

DISCUSSION

Despite being commonly invoked as radical-trapping antioxidants, thiols are not competent in this regard. Their reactions with peroxyl radicals are not thermodynamically favorable (essentially thermoneutral), and the kinetics ($<10^3$ M^{-1} s^{-1}) are too slow to compete with chain propagation, particularly given the low concentration and/or poor solubility of most thiols in lipid bilayers.[61,62] Nevertheless, increasing the availability of thiols, for example, via treatment with *N*-acetylcysteine (NAC), can have an impact on cellular lipid peroxidation and the cell's resistance to ferroptosis.[63−65] At first glance, the obvious mechanism would appear to be an increased pool of reduced glutathione, which serves as the reducing cosubstrate for the glutathione peroxidases (GPXs). However, it is plausible that other (GPX-independent) mechanism(s) contribute. Dick and co-workers recently showed that NAC treatment leads to an increase in cellular RSSH via the transsulfuration pathway.[65,66] Our recent finding that RSSH are excellent H-atom transfer agents, reacting in highly exergonic reactions with peroxyl radicals with very fast kinetics,[22] prompted us to investigate whether they can inhibit (phospho)lipid peroxidation and associated ferroptosis as RTAs.

The experiments we have carried out with select alkyl hydropersulfides clearly demonstrate they are indeed potent

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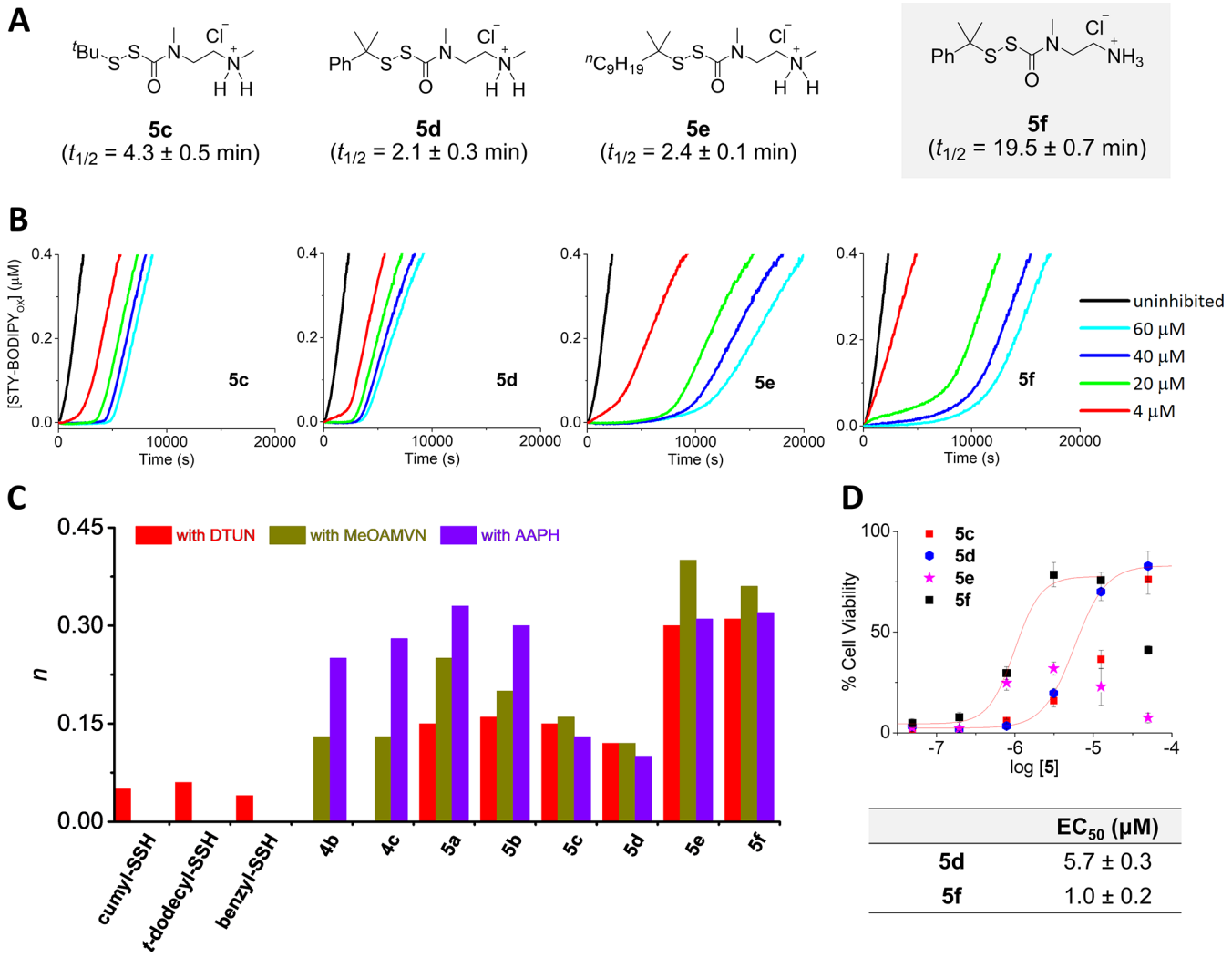

Figure 7. (A) *N*-Alkylamine-substituted perthiocarbamates **5c**–**f** that lead to *t*-butyl, cumyl, and *t*-dodecyl hydropersulfides. (B) Representative co-autoxidations of STY-BODIPY (1 μM) and PC liposomes (1 mM) in PBS (10 mM) at pH 7.4 initiated by DTUN (200 μM) at 37 °C and inhibited by RSSH precursors **5c**–**f**. Reaction progress was monitored by fluorescence (λ_{ex} = 488 nm, λ_{em} = 518 nm). (C) Radical-trapping stoichiometries of authentic RSSH and RSSH precursors (20 μM) from inhibited co-autoxidations initiated by DTUN (200 μM), MeOAMVN (200 μM), or AAPH (1 mM) at 37 °C. (D) Rescue of RSL3-induced ferroptosis in Pfa1 MEFs by RSSH precursors **5c**–**f** and corresponding EC$_{50}$ values of **5d** and **5f**.

inhibitors of (phospho)lipid peroxidation. In fact, their peroxyl radical-trapping kinetics are similar to those of phenoxazine (i.e., $k_{inh} > 10^5$ M^{-1} s^{-1}), the most potent inhibitor characterized to date.[30,32] However, the efficacy of RSSH is limited by their instability; in aqueous solutions where RSSH (p$K_a \sim 7$)[22] is in equilibrium with its conjugate base, they are known to rapidly decompose to form H_2S, thiols, and polysulfides. As such, while RSSH consistently trap one equivalent of peroxyl radical in organic solvent, in liposomal suspensions only small fractions of this stoichiometry are observed. This is consistent with results we previously obtained in aqueous solution; while ionization of RSSH to the perthiolate slightly enhances its reactivity with peroxyl radicals (revealed by a marginal increase in k_{inh} with increasing pH) due to the contribution of a single electron transfer mechanism, it is associated with diminishing *n*.[74] Corresponding experiments in either aqueous solution or liposomal suspensions with RSSH-derived decomposition products reveal little to no inhibitory activity, implying that, although unstable, RSSH are likely the most reactive sulfur-based inhibitors of lipid peroxidation in the cell.[75] The fact that H_2S_2 and RSSH derived from their reaction with thiols (e.g., penicillamine-SH and glutathione) possess RTA activities reinforces the contention that RSSH, and not H_2S or RSH, effectively suppress lipid peroxidation.

Due to the intrinsic instability of RSSH in aqueous solutions, model studies of their reactivity have benefitted from small molecules that are able to slowly and steadily release them, minimizing their decomposition under relevant conditions.[17,19,47] The reactivity of the three types of precursors studied above depended significantly on the polarity of the initiator of (phospho)lipid peroxidation. When DTUN, the lipophilic hyponitrite typically used in the FENIX assay, was used, only the *N*-alkylamine perthiocarbamates displayed significant activity. However, when the amphiphilic MeOAMVN or hydrophilic AAPH initiators were used, the *S*-alkylsulfenyl isothioureas also displayed significant activity. Since RSSH formation from these precursors requires their deprotonation, they are presumably formed at the lipid–water interface, where they can trap initiator-derived radicals. On the

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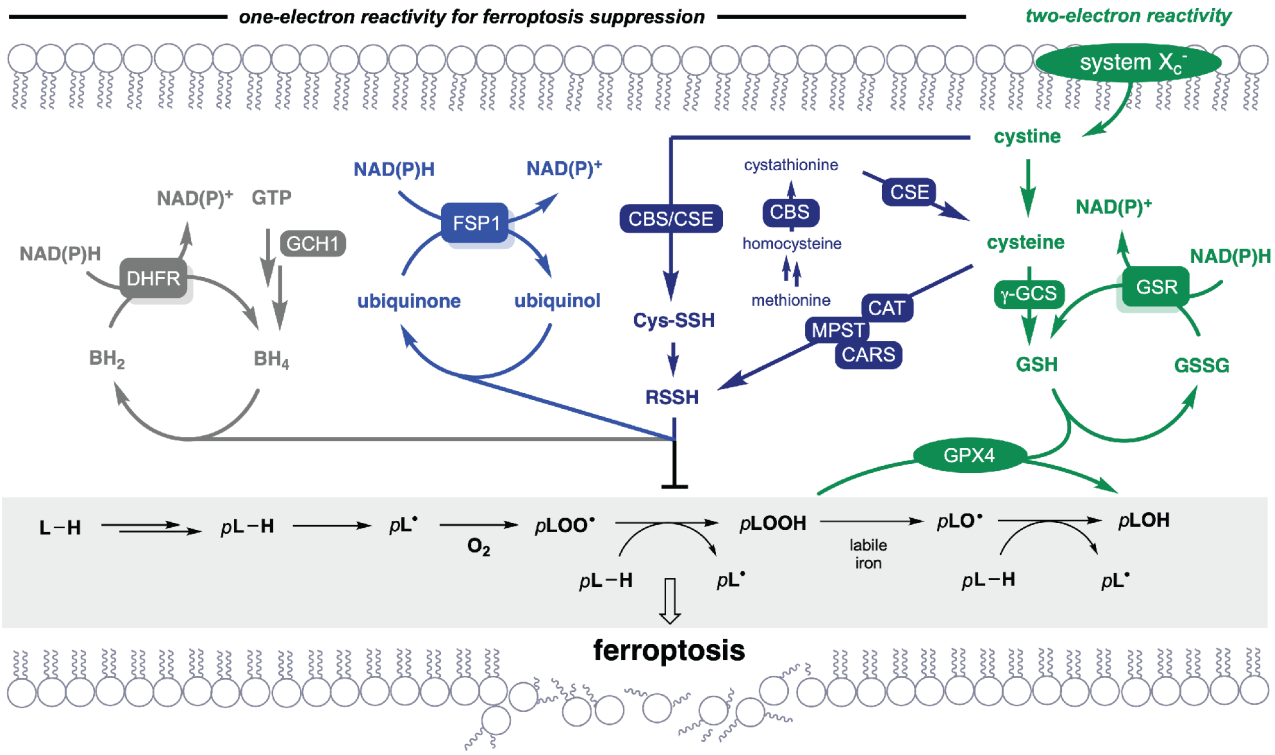

Figure 8. Simplified model of ferroptosis suppression systems delineating one-electron (radical-trapping antioxidant) mechanisms for inhibition of phospholipid (*p*L-H) peroxidation from the two-electron mechanism exhibited by the master regulator GPX4.

other hand, the perthiocarbamates do not need to partition to the aqueous phase and can release RSSH via an intramolecular substitution (cyclization) in proximity to the lipid phase, enabling them to more efficiently trap (phospho)lipidperoxyl radicals. The more slowly releasing *S*-alkylsulfenyl thiocarbonates were not effective inhibitors, regardless of the polarity of the radical initiator. We surmise that, instead of hydropersulfides, RSS-derived polysulfides were the major products of these precursors, presumably formed from reactions between RSSH and the thiocarbonate moieties of the precursors, a liability identified in their design and characterization.[50]

Previous efforts from our lab have established that inhibition rate constants determined from DTUN-initiated liposomal autoxidations provide good quantitative predictions of the potency of small molecules in ferroptotic cell death rescue assays.[43] While quantitative predictions from the current studies are confounded by the instability of RSSH, the results nonetheless correctly foretell that authentic RSSH and the *N*-alkylamine perthiocarbamate precursors will rescue cells from ferroptosis. Of the RSSH tested, the cumyl derivative was clearly most effective (EC_{50} = 6 μM), with the benzyl and *t*-dodecyl hydropersulfides failing to rescue to >50% before displaying toxicity that can be attributed to their decomposition to polysulfides, which are toxic at elevated concentrations.[67] The *N*-alkylamine perthiocarbamates were less potent (EC_{50} ~ 18 μM), perhaps due to the lower lipid solubility of the *N*-acetylpenicillamine-derived hydropersulfide. To probe this point, new alkylamine-substituted perthiocarbamates leading to cumyl, *t*-butyl, and *t*-dodecyl hydropersulfides were prepared (structures **5c**−**e** in Figure 7A, see Supporting Information for complete details). We found these precursors to be similarly reactive to the authentic RSSH, fully suppressing lipid peroxidation in liposomes (Figure 7B), but with superior efficiency (i.e., radical-trapping stoichiometries), as summarized in Figure 7C. Moreover, cells were better rescued from ferroptosis with these precursors (Figure 7D, EC_{50} = 5.7 μM for **5d**) than those giving rise to the less hydrophobic *N*-acetylpenicillamine-derived hydropersulfides. This is best illustrated via comparison of the results obtained with **5d** to **5b** (EC_{50} ~ 18 μM), which had a similar half-life for RSSH release. Unfortunately, despite showing greater cell rescue at low dose, the superior reactivity of the most lipophilic precursor **5e** in liposomes did not translate to cells, presumably due to associated lipotoxicity (see Supporting Information).

The observation that cumyl-SSH precursor **5d** possessed essentially equivalent potency to that of cumyl hydropersulfide implied no advantage to the precursor strategy in this context, a fact belied by its very short half-life (<2 min). Although this appears sufficient to provide an advantage in liposomes (n ~ 0.3 for **5d** vs n < 0.05 for **1**), cell incorporation is presumably slower and/or less efficient due to reactions of RSSH and/or the precursors with media components. Given the longer lifetime of the primary amine-terminated precursor **5a** ($t_{1/2}$ = 16.7 min) relative to the secondary amine-terminated precursor **5b** ($t_{1/2}$ = 1.4 min), we prepared precursor **5f** (Figure 7A, see Supporting Information for details). In the event, the half-life of **5f** was extended to 20 min, and, most gratifyingly, this led to an improvement in potency of almost 1 order of magnitude (EC_{50} ~ 1 μM) (Figure 7D). It stands to reason that with further optimization for precursor half-life, lipophilicity, and stability, using the preliminary structure−reactivity insights provided here, it will be possible to increase the potency of hydropersulfide precursors as ferroptosis inhibitors. Such efforts may provide leads to therapeutics for indications in which ferroptosis has been implicated.

Although the antiferroptotic potency of RSSH and their precursors is far lower than the archetype inhibitors Fer-1 and

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Lip-1, it is important to consider that RSSH are produced endogenously. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) of the transsulfuration pathway produce CysSSH either directly from cystine or H_2S from cysteine, which can then be utilized by sulfide:quinone oxidoreductase to produce RSSH. H_2S-independent RSSH formation can arise via deamination of cysteine by cysteine aminotransferase (CAT) and subsequent desulfuration of mercaptopyruvate by the associated sulfurtransferase (MPST) to form an MPST-bound hydropersulfide that can be exchanged with other thiols. Most recently, cysteinyl−tRNA synthetase (CARS) has been proposed to convert cysteine to its hydropersulfide and incorporate CysSSH into protein during translation.[68−70] The identity/identities of RSSH that may act to suppress ferroptosis in the cell is unclear and should be the subject of future study. Although CysSSH and GSSH are not particularly lipid-soluble, they are likely to be most abundant, and some may partition to the lipid phase and/or react with lipid-derived peroxyl radicals at or near the lipid/aqueous interface. Indeed, we show above that GSH and H_2S_2 exhibit synergy in the inhibition of DTUN-initiated lipid peroxidation, which suggests that GSSH can at leastand partially partition to the lipid phase/interface.

Given the multiphasic dose−response of H_2S and RSSH (cytoprotective at low concentrations and cytotoxic at high concentrations), their formation and utilization are highly regulated. As such, in many ways, RSSH may be viewed as another regulated ferroptosis suppression system, akin to GCH1/BH_4. Upregulation of GTP cyclohydrolase-1 (GCH1) has been found to confer ferroptosis resistance via its role as catalyst of the rate-limiting step of tetrahydrobiopterin (BH_4) synthesis.[37,38] Like RSSH, BH_4 is a potent RTA, but is highly unstable, requiring its *de novo* synthesis to be tightly regulated. Also, like RSSH, BH4 is not particularly potent when added directly to cell culture due to its instability, necessitating its endogenous regeneration by dihydrofolate reductase (DHFR) at the expense of the cell's universal stoichiometric reductant, NAD(P)H.[38] Thus, it is tempting to suggest that RSSH formation should be considered a fourth endogenous ferroptosis suppression system (Figure 8).

Cysteine availability has been at the center of ferroptosis since before its definitive characterization. Early work from Stockwell established that the targets of erastin[71] and RSL3,[58,59] the archetype inducers of ferroptosis, were system x_c^- and GPX4, respectively, establishing the cystine/GSH/GPX4 axis as the master regulator of ferroptosis. More recently, multiple reports have implicated the transsulfuration pathway in ferroptosis suppression, as it can provide an alternative source of cysteine for GSH biosynthesis to compensate for system x_c^- inhibition/dysfunction. For example, upregulation of CBS conferred erastin resistance to CARS-deficient cells.[72] While maintaining the supply of cysteine for GSH biosynthesis to fuel GPX4 activity is an obvious contributor to ferroptosis suppression in these contexts, our results suggest that hydropersulfide production could also play a role. Notably, Stockwell found that CARS knockdown did not suppress RSL3-mediated ferroptosis, but this may be because induction of CBS is further enhanced by erastin treatment, due to cystine starvation. Indeed, prolonged erastin treatment alone has been reported to induce ferroptosis resistance driven by sustained upregulation of CBS.[73] In light of these observations and our own chemical investigations, it seems further work on the role of gene products involved in the synthesis/reactions of RSSH (and related persulfide species) in ferroptosis suppression is warranted.

CONCLUSION

We had previously shown that RSSH possess impressive H-atom transfer reactivity, driven by both thermodynamics (weak S−H bond) and kinetics (favorable polar/secondary orbital interactions in the transition state). We have now shown that this impressive reactivity extends to the confines of lipid bilayers, where they are able to trap (phospho)lipid peroxyl radicals with kinetics similar to those of the best inhibitors reported to date. The efficiency of radical trapping is limited by the instability of RSSH in aqueous media, a reality that can be somewhat circumvented when they are produced from appropriate precursors *in situ*. *N*-Alkylamine perthiocarbamates are particularly useful in this regard, as they do not depend on hydrolytic reactions for RSSH generation, but instead on intramolecular substitution. Authentic RSSH were found to inhibit ferroptosis induced by either GPX4 inhibition or *gpx4* deletion, and their potency in cells could be augmented when they were formed from appropriate precursors *in situ*. From these studies it is clear that RSSH can be employed to inhibit lipid peroxidation via their action as radical-trapping antioxidants. Given the ability to synthesize RSSH on demand, cells may upregulate RSSH production when necessary as a ferroptosis suppression mechanism.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c06804.

Materials and methods, additional inhibited autoxidation data and corresponding derived kinetics values, additional cell rescue and cytotoxicity data, synthetic procedures, and corresponding NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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(74) We cannot explicitly rule out the contribution of an electron-transfer pathway in the liposomal suspensions. In homogeneous solution, we observed that hydrogen−deuterium kinetic isotope effects on k_{inh} disappear upon moving from organic to aqueous solution, consistent with a mechanism change from H-atom transfer from RSSH to electron transfer from RSS^-.[22] Analogous mechanistic studies in the aqueous liposomal suspensions used in the current work were made difficult by the complete suppression of the rate of lipid peroxidation by RSSH and the very short inhibition periods observed. Nevertheless, it seems reasonable to suggest that the major reaction pathway in liposomes is H-atom transfer given that the lipophilicity of the RSSH and/or its precursor significantly impacts its reactivity.
(75) It must be acknowledged that we have not investigated higher hydro(poly)sulfides (RS_nSH, $n > 1$), and these may arise under some of the conditions we have investigated. Given that the stabilities of perthiyl (RSS•) and related polysulfide-derived radicals (RS_nS•) are known to be similar,[52] it is expected that hydropolysulfides (RS_nSH) will have similar H-atom transfer reactivity to hydropersulfides (RSSH).

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