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Discoveries

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Methods for Suppressing Hydrogen Sulfide in Biological Systems

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Abstract

Significance: Hydrogen sulfide (H_2S) plays critical roles in redox biology, and its regulatory effects are tightly controlled by its cellular location and concentration. The imbalance of H_2S is believed to contribute to some pathological processes.
Recent Advances: Downregulation of H_2S requires chemical tools such as inhibitors of H_2S-producing enzymes and H_2S scavengers. Recent efforts have discovered some promising inhibitors and scavengers. These advances pave the road toward better understanding of the functions of H_2S.
Critical Issues: Precise H_2S downregulation is challenging. The potency and specificity of current inhibitors are still far from ideal. H_2S-producing enzymes are involved in complex sulfur metabolic pathways and ubiquitously present in biological matrices. The inhibition of these enzymes can cause unwanted side effects. H_2S scavengers allow targeted H_2S clearance, but their options are still limited. In addition, the scavenging process often results in biologically active by-products.
Future Directions: Further development of potent and specific inhibitors for H_2S-producing enzymes is needed. Scavengers that can rapidly and selectively remove H_2S while generating biocompatible by-products are needed. Potential therapeutic applications of scavengers and inhibitors are worth exploring. *Antioxid. Redox Signal*. 36, 294–308.

Keywords: hydrogen sulfide, inhibitor, scavenger, cystathionine-β-synthase, cystathionine-γ-lyase, 3-mercaptopyruvate sulfur transferase

Introduction

Hydrogen sulfide (H_2S) is the newest member of the gasotransmitter family, which also includes nitric oxide (NO) and carbon monoxide (CO). Studies have shown that H_2S regulates various physiological and pathological responses (22, 44, 62, 74). The first notable function of H_2S in mammalian systems was discovered by Abe and Kimura (1), which demonstrated H_2S as a neuromodulator in the brain. Following this seminal work, many other functions of H_2S (such as antioxidation, anti- or proinflammation, and vasodilation) were reported. H_2S is a reactive and diffusible molecule, and it can be further converted into other reactive sulfur species in biological systems. The functions of H_2S are believed to be the result of its concentration, location, and biochemical reactions (7, 19, 33, 35). Dysregulated H_2S exerts disparate impacts in pathological processes, such as cancer, inflammation, and cardiovascular diseases (13, 70). In this regard, the development of H_2S regulating methods or tools has become an important research field, and a large number of such tools have been reported. So far, some excellent review articles have been published, which cover the design, mode of action, properties, and applications of H_2S-releasing or supplying agents (*i.e.*, prodrugs or donors) (34, 53, 81, 83). On the contrary, reviews on H_2S-suppressing agents are relatively rare. Herein, we summarize reports on

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H_2S-downregulating agents, mainly focusing on inhibitors of H_2S-producing enzymes and H_2S scavengers. Their design principle, activity, applications, and potential limitations are discussed.

Biosynthesis of H_2S

Up until now, at least four enzymes (cystathionine-β-synthase [CBS], cystathionine-γ-lyase [CSE], 3-mercaptopyruvate sulfur transferase [3-MST], and cysteinyl-tRNA synthetases [CARS]) are believed to contribute to the production of H_2S in mammalian systems (Fig. 1). The expressions of H_2S-producing enzymes are tissue specific. For example, CBS is the predominate source of H_2S in liver, brain, and nervous tissues (20, 54, 55). CBS is a pyridoxal-5′-phosphate (PLP)-dependent enzyme, which catalyzes the conversion of $_L$-homocysteine or $_L$-cysteine to $_L$-cystathionine, lanthionine, $_L$-serine, and H_2S in cytoplasm. CSE is critical for H_2S production in vasculature. It catalyzes PLP-dependent reactions converting $_L$-homocysteine, $_L$-cystathionine, and $_L$-cysteine to α-ketobutyrate or pyruvate and releases H_2S. It should be noted that CBS and CSE are important enzymes in metabolism, and they are involved in other critical transformations. For example, a primary role of CBS is to convert $_L$-serine and $_L$-homocysteine to form $_L$-cystathionine. Therefore, the significance of CBS/CSE should not just be attributed to H_2S formation. CBS and CSE are predominantly located in the cytoplasm. However, under some conditions of stress, they can translate to nuclei or mitochondria (19, 21, 65).

3-MST is expressed in both cytoplasm and mitochondria. In cooperating with cysteine aminotransferase (CAT), 3-MST catalyzes H_2S formation by employing $_L$-cysteine as the substrate. In this process, CAT first converts $_L$-cysteine to 3-mercaptopyruvate (3-MP) with α-ketoglutarate as the co-substrate (Fig. 1c). Then, 3-MP transfers its sulfur to the cysteine residue of 3-MST to form 3-MST persulfide, which finally reacts with thioredoxin or thiols to produce H_2S. $_D$-Cysteine can also be catalyzed by 3-MST to release H_2S with the help of $_D$-amino acid oxidase (DAO). Again, 3-MP is the key intermediate. However, this is not a physiological production of H_2S as $_D$-cysteine needs to be exogenously administered. CARS is the most recently discovered H_2S-releasing enzyme (4). The primary products of CARS are cysteine persulfide and polysulfides, which are unstable species and can easily be converted to H_2S, especially in the presence of cellular thiols (Fig. 1d). As such, H_2S can be considered as a downstream product from CARS reactions, and CARS is at least partially responsible for H_2S biosynthesis. The two different isoforms of CARS, CARS1 and CARS2, are found in mitochondria and cytoplasm, respectively.

In addition to these enzyme-mediated generations, H_2S can be produced in nonenzymatic pathways (Fig. 2). For example, highly reactive sulfane sulfur species such as allyl trisulfide can readily react with glutathione (GSH) in red blood cells to form H_2S (6). Less reactive sulfane sulfur species, such as elemental sulfur (S_8), are not very reactive toward GSH. However, in human erythrocytes S_8 can be effectively converted to H_2S by cellular reducing equivalents

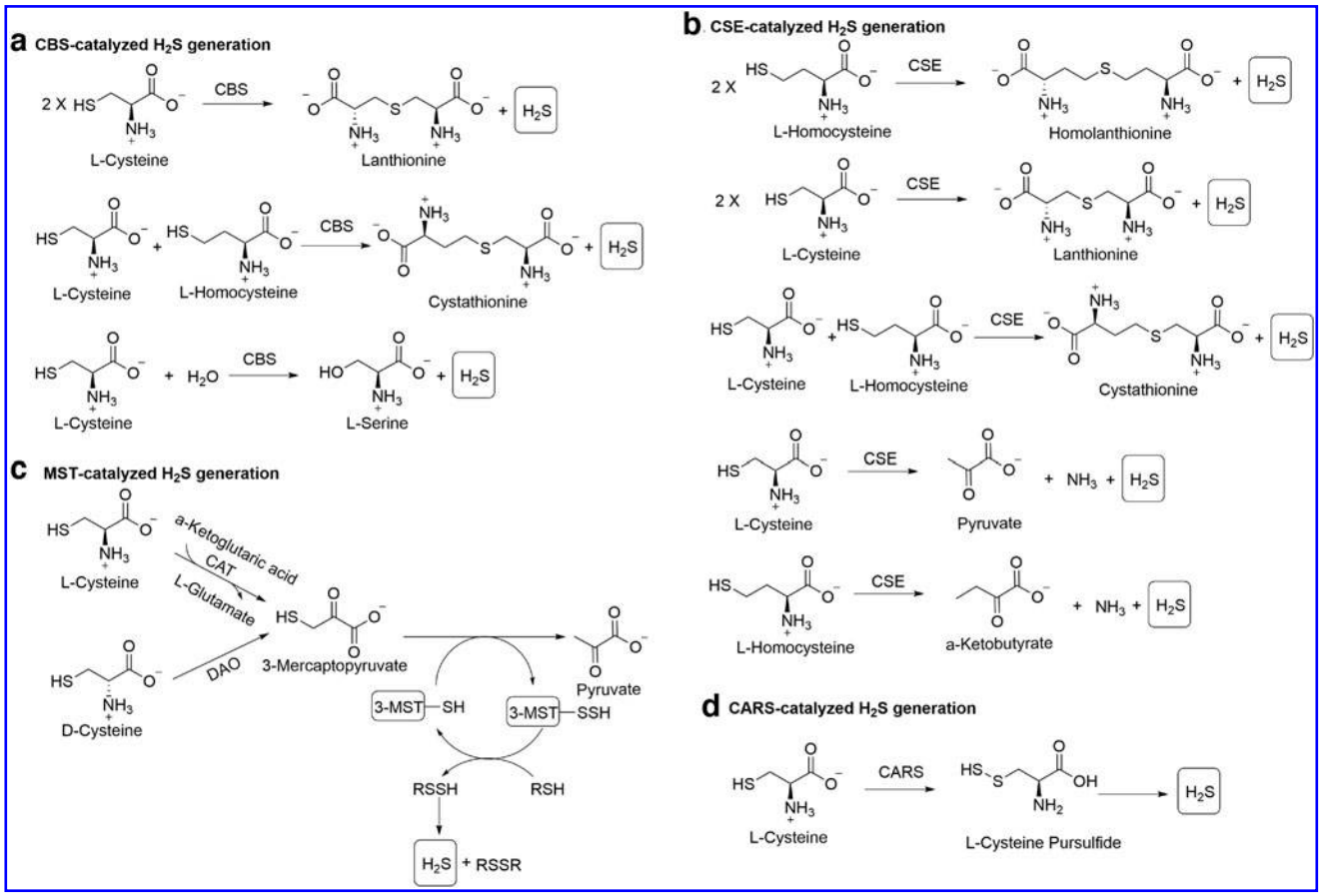

FIG. 1. Enzyme-mediated H_2S generation. H_2S, hydrogen sulfide.

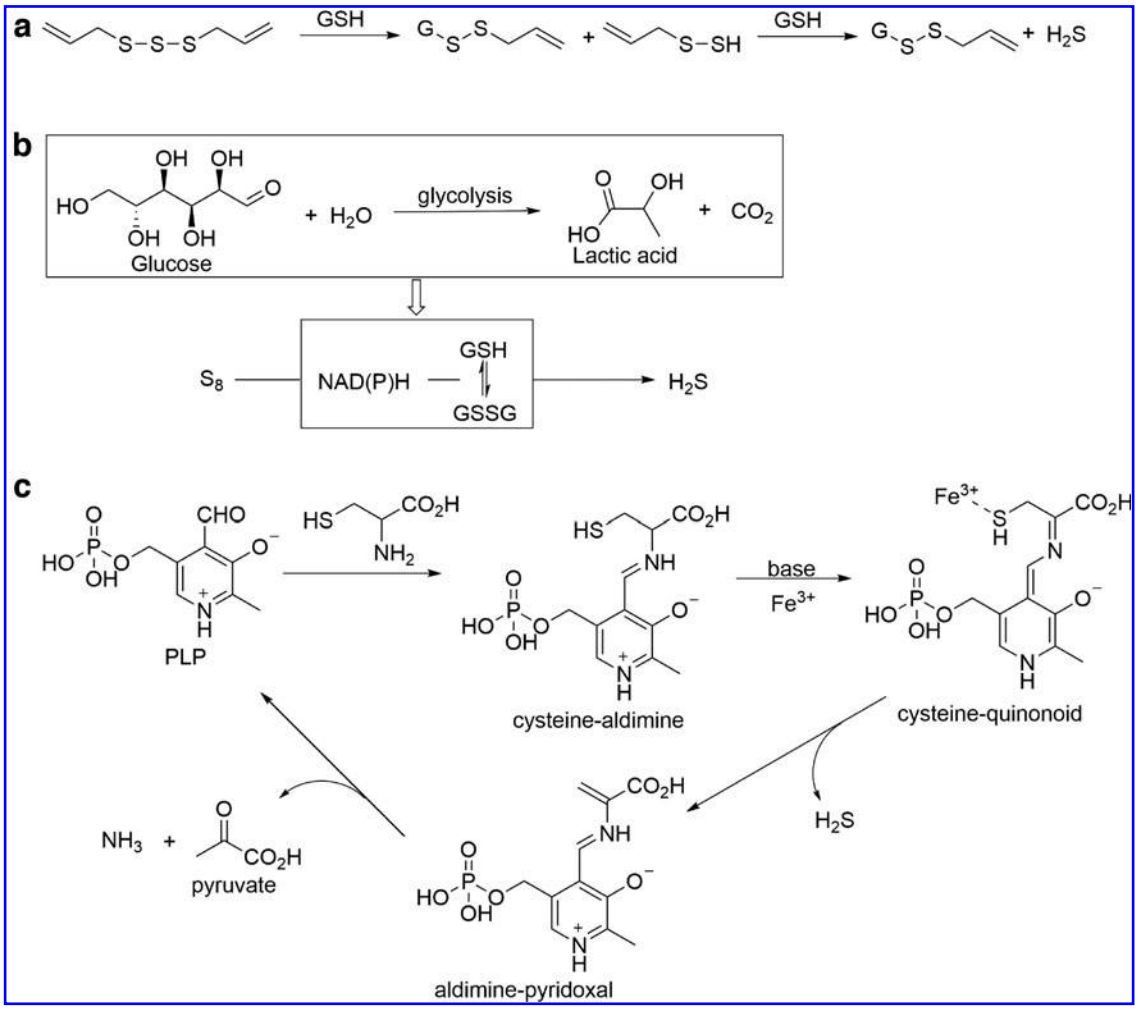

FIG. 2. Nonenzyme-mediated H_2S generation.

obtained from glucose oxidation (*e.g.*, glucose glycolysis) (59). These reducing equivalents are found to be nicotinamide adenine dinucleotide phosphate (NADPH)/nicotinamide adenine dinucleotide (NADH), in conjugation with GSH. While the detailed reaction mechanism is still unclear, it was found that combinations of GSH and NAD(P)H were synergistic. It should be noted that allyl trisulfide and elemental sulfur are normally given exogenously (such as from food). Therefore, these pathways are not considered as physiological ways of H_2S production. Very recently, it was discovered that cysteine (both $_D$- and $_L$-) could be converted to H_2S *via* coordinated catalysis by vitamin B_6, pyridoxal(phosphate), and iron under physiological conditions (80). Initially, cysteine-aldimine is formed between cysteine and the pyridoxal (phosphate) aldehyde. Then, iron derives the formation of cysteine-quinonoid, -SH elimination, and hydrolysis of desulfurated aldimine. Eventually this reaction produces pyruvate, ammonia (NH_3), and H_2S.

H_2S Catabolism

The accumulation of H_2S could cause severe organ toxicity. In mammalian systems, currently known H_2S catabolic pathways include oxidation, methylation, and scavenging by metallo- or sulfur-containing macromolecules. In mitochondria, a series of enzymes including sulfide quinone oxidoreductase, sulfurdioxygenase, and rhodanese catalyze H_2S oxidation to form thiosulfate, sulfite, and sulfate sequentially (36). As the end-product, sulfate can be disposed by urinary excretion. In addition to these enzymes, ferric hemoglobin and myoglobin can promote H_2S oxidation to form thiosulfate and iron-bound persulfide species (9). H_2S methylation mainly occurs in the cytosol. Catalyzed by thiol S-methyltransferase, H_2S is first converted to methanethiol and then to nontoxic dimethyl sulfide. Compared with oxidation, this process is much slower (37). Therefore, it is considered a minor H_2S catabolism pathway. Finally, H_2S can also react with macromolecules such as hemoglobin to form H_2S bound species such as sulfhemoglobin (14). This may serve as an internal storage of H_2S. It should be noted that these H_2S catabolism pathways, especially its oxidation in mitochondrial, do not simply occur as a way for H_2S detoxification. These are physiological pathways normally happening in cells. For example, the mitochondrial oxidation is critical in cellular bioenergetics as H_2S serves as an electron donor for electron transport chain.

H_2S in Pathological Processes

Understanding the steady state of H_2S in circulation is still a challenge. This is mainly due to the high reactivity toward molecular oxygen and the high volatility of H_2S. In complex

biological environments, such as live cells and tissues, the presence of other reactive sulfur species—especially biothiols and persulfides—often results in spuriously high H_2S concentrations. In addition, the methods used in H_2S measurement can make a difference as they tend to have varied sensitivity and selectivity (46, 76). Because of these constraints, the steady level of endogenous H_2S and its ideal therapeutic concentration are controversial. It is now accepted that H_2S acts as a double-edged sword in pathological processes. For example, cytoprotective effects of H_2S were found in cardiac diseases, asthma, and reperfusion injury. In a myocardial ischemia–reperfusion injury murine model, the generation of H_2S was deficient. H_2S-releasing compounds such as JK-1 could be used to restore cellular H_2S levels and exhibit promising cardioprotective effects (32). On the contrary, overproduction of H_2S can induce adverse impacts in cancer, inflammation, and even hypertension. Compared with noncancerous cells, the rate of H_2S generation in colon cancer-derived cells was much higher (26). By silencing CBS, the growth of colon cancer and blood flow around tumors was found to be suppressed in nude mice (63). In a burn-induced inflammation model, H_2S generation was upregulated in plasma and liver. Different from the control (saline injected) and propargylglycine (PAG, CSE inhibitor)-injected mice, burned mice with injected NaHS solution exhibited significantly aggravated systemic inflammation (82). Furthermore, H_2S is known as a cyanide-like highly toxic substance. It inhibits cytochrome C oxidase in electron transfer chain and affects adenosine triphosphate (ATP) synthesis (49). Short-time exposure to high levels of H_2S can lead to irreversible neurologic, respiratory, and cardiovascular deficits (56). While H_2S poisoning has been a concern for many years, specific H_2S antidotes are still lacking. On the contrary, H_2S at physiological concentration was found to directly reduce cytochrome C and produce a $HS^{\bullet}/S^{\bullet-}$ radical. This process could stimulate protein persulfidation, and thus plays an important role in H_2S signaling (69).

Inhibitors of H_2S-Producing Enzymes

CBS inhibitors

Aminooxy (R-ONH_2)-based CBS inhibitors. Aminooxyacetic acid (AOAA) is one of the most well-known inhibitors of CBS. The understanding of AOAA's responding mechanism started with the discovery of the reaction between AOAA and vitamin B_6. In 1961, McCormick and Snell found that AOAA was able to react with the aldehyde group of vitamin B_6 (pyridoxal form) and generate a stable oxime **1** in aqueous solutions (Fig. 3) (37). PLP is the metabolically active form of vitamin B_6, which participates in a series of enzymatic reactions in biological systems. PLP is the cofactor of CBS. Through embedding PLP into the active center, the Schiff base **2** is generated. After a series of additions and eliminations, H_2S is formed from cysteine or homocysteine. While in the presence of AOAA, oxime **3** is generated irreversibly and therefore, inhibits CBS-catalyzed

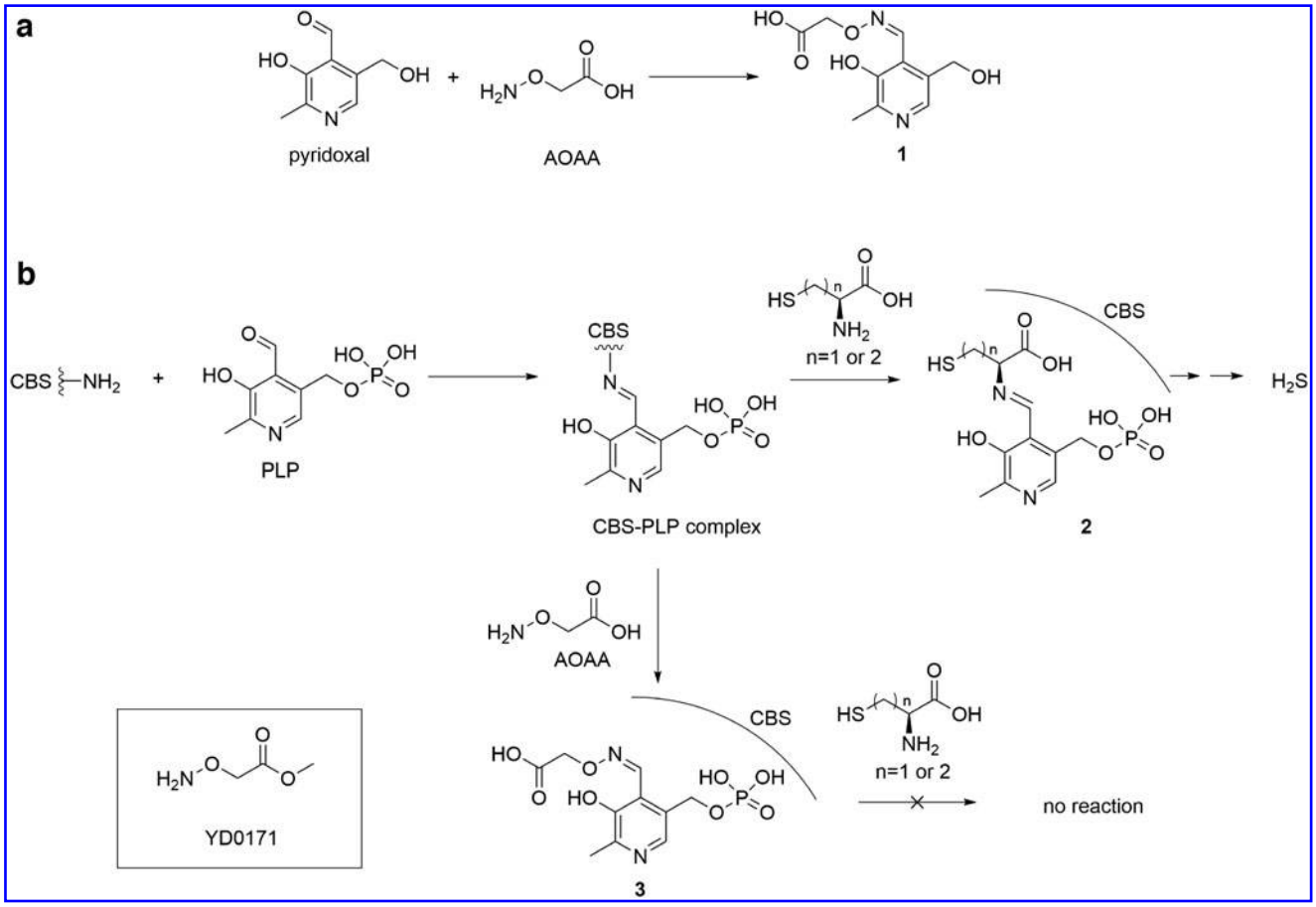

FIG. 3. Inhibitory mechanism of AOAA and the structure of YD0171. AOAA, aminooxyacetic acid.

H_2S generation. In sodium phosphate buffer, half-maximal inhibitory concentration (IC_{50}) of AOAA on CBS is $8.52 \pm 0.71\ \mu M$ (5). AOAA has been wildly used in the past years. For example, in the construction of H_2S detection platform HSN2-BG, Montoya and Pluth employed AOAA to down regulate the production of endogenous H_2S in cytoplasm of Chinese hamster ovary (CHO) cells (40). Zhao and colleagues treated HCT116 cells with AOAA to establish the negative control for examining the detection ability of a cancerous cell-targeted H_2S probe (75). Previous research indicated that the expression of CBS in Down syndrome cells (DSCs) was higher than that in normal cells. Szabo and colleagues used AOAA to silence CBS in DSCs. They discovered that by normalizing H_2S levels, mitochondrial electron transport and proliferation ability of DSCs were restored (47). Even with these applications, it is worthy to note that AOAA is not a specific inhibitor for CBS. About 40 PLP-dependent enzymes, such as decarboxylase, aminotransferase, and racemase, have been found to be affected by AOAA in either *in vitro* or *in vivo* tests (24, 28, 78). Interestingly, Asimakopoulou *et al.* indicated that even though AOAA was always considered as an inhibitor for CBS, the inhibitory efficiency of AOAA on CSE ($IC_{50} = 1.09 \pm 0.12\ \mu M$) was tested to be stronger than that of CBS (5). Another drawback of AOAA is the low lipophilicity (water/octanol coefficient: 0.0019). While in aqueous solutions the IC_{50} of AOAA for CBS is at low micromole level, its efficiency significantly drops in cells due to poor lipophilicity. In HCT116 colon cancer cells, up to $100\ \mu M$ AOAA was needed to induce noticable inhibition of H_2S generation, presumably due to low cellular uptake (43).

To improve its lipophilicity, a prodrug of AOAA, YD0171, was developed by Szabo and colleagues (15). Compared with AOAA, YD0171 showed weaker inhibition activity toward CBS ($IC_{50} = 300\ \mu M$). However, YD0171's lipophilicity was enhanced to 0.121, 63.8-fold higher than AOAA. After penetrating cell membrane, the ester linkage on YD0171 can be easily hydrolyzed by cellular esterase to form AOAA. As such, the efficiency of YD0171 was improved. CBS inhibition caused H_2S downregulation upon treating HCT116 cells with $30\ \mu M$ YD0171, while $100\ \mu M$ AOAA was needed for similar effects. In animal models, injecting 1 mg/kg/day of YD0171 into mice for 3 weeks led to the volume suppression of xenograft colon tumor by 90% (similar results were achieved by injecting 9 mg/kg/day of AOAA during the same period). Furthermore, YD0171 was shown to be safer than AOAA. The mortality in mice injected with YD0171 (1 mg/kg/day) was 14% after 21 days, while the mortality of AOAA (9 mg/kg/day)-injected mice was up to 53%. The inhibitory effects of YD0171 were found to be selective for cancer cells. The expression of CBS was not limited to colon tumor. Compared with the inhibitory effects on colon cancer proliferation, the effects of YD0171 on hepatocytes were negligible, which was due to either fast metabolism in hepatocytes or different turnover rates of CBS in tumor cells *versus* hepatocytes. Like AOAA, YD0171 is a nonspecific inhibitor for PLP-dependent enzymes. Its inhibition on other PLP-dependent enzymes, such as glutamic oxaloacetic transaminase 1 (GOT1), may account for the suppression of cancer growth as well.

In addition to AOAA and YD0171, the simplest compound bearing the aminooxy moiety, hydroxylamine (NH_2OH), has been considered as a CBS inhibitor. The original use of hydroxylamine was to separate ketones and aldehydes from biological matrix (12). By undergoing imine formation, hydroxylamine can convert water-soluble ketones and aldehydes to insoluble oximes, which then precipitate out from cell lysates. Based on this reactivity, hydroxylamine was used in deactivating aldehyde-contained PLP-dependent enzymes (5). Although it showed some inhibitory activity on CBS ($IC_{50} = 278.0 \pm 22.0\ \mu M$), the application of hydroxylamine as a CBS inhibitor was still limited. This was because (i) hydroxylamine showed higher inhibition activity toward CSE ($IC_{50} = 4.83 \pm 0.31\ \mu M$); (ii) hydroxylamine was unstable in aqueous solution. At room temperature (25°C), hydroxylamine can decompose to form a series of reactive nitrogen species, such as nitrous oxide (N_2O), NH_3, and tiny amount of NO (73). Almost all these nitrogen species have their own unique biological functions. Therefore, the use of hydroxylamine as a CBS inhibitor can be compromised.

Hydrazine (H_2NNH_2)-based CBS inhibitors. Some hydrazine derivatives, such as isoniazid, are known as antibiotic drugs for treating tuberculosis. However, high-dose isoniazid can cause a series of vitamin B_6 antagonism-related side effects, including pellagra, convulsion, and peripheral neuropathy (51). These findings inspired the study of inhibitory effects of hydrazine derivatives on PLP-dependent enzymes. A hydrazine derivative 2,3,4-trihydroxylbenzylhydrazine (THBH) was found to be a CBS inhibitor ($IC_{50} = 30\ \mu M$) (18). In HCT116 cells, obvious inhibition in proliferation was noted when treating with 30–100 μM THBH for 48 h. The inhibition mechanism of THBH was studied by structural modeling. The formation of a PLP-THBH hydrazone derivative **4** (Fig. 4) was found to be favored. In the same way, 3-hydroxylbenzylhydrazine was also used as a CBS inhibitor.

Benserazide, a THBH derivative, was found to be another CBS inhibitor (66). Compared with the control (containing CBS, $_L$-cysteine, homocysteine, and PLP), H_2S generation in the benserazide-treated group was significantly decreased. Under the same conditions, H_2S formation from a donor GYY4137 was not affected by benserazide, indicating that benserazide downregulated H_2S concentration by inhibiting CBS, not by scavenging H_2S. In biological systems, benserazide could degrade (by hydrolysis or deacylation) to form THBH and thus achieve inhibition. However, benserazide also contains a free amine group, which could conjugate with the PLP aldehyde to form a Schiff base and cause inhibition. Docking studies revealed that the latter was favored. Benserazide is not a potent inhibitor as its IC_{50} toward CBS (in buffers) is only $30\ \mu M$, much weaker than AOAA. However, in cell-based studies its IC_{50} was shown as $20\ \mu M$, which could be attributed to good cellular uptake (18). Under standard conditions (2 mM $_L$-cysteine and homocysteine for CBS; 10 mM $_L$-cysteine for CSE; 10 mM 3-MP for 3-MST), benserazide exerted some selectivity toward CBS. Up to 66% CBS activity was inhibited after treating $100\ \mu M$ benserazide for 2 h. Under the same conditions, the inhibitory effects on CSE and 3-MST were only 16% and 35%, respectively. It should be noted that the inhibitory effect of benserazide was related to the concentration of substrates. Using CBS as an example, when the concentrations of $_L$-cysteine and homocysteine (substrates of CBS) were increased to 10 mM, the inhibitory effect of benserazide ($100\ \mu M$) was decreased to

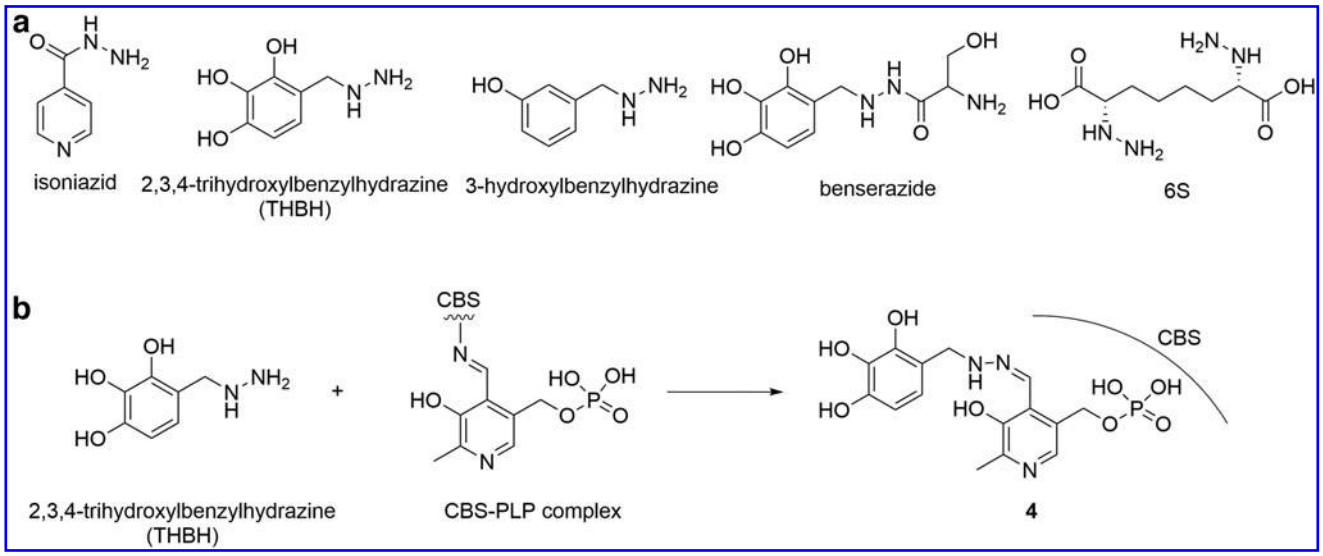

FIG. 4. Hydrazine-based CBS inhibitors and inhibitory mechanism of THBH. CBS, cystathionine-β-synthase; THBH, 2,3,4-trihydroxylbenzylhydrazine.

10% ± 2%. Therefore, one should not draw the conclusion that benserazide showed selectivity toward CBS by simply comparing inhibitory effects when concentrations of substrates are different.

In 2016, Berkowitz and colleagues reported a ''zipped synthesis'' approach for the development of CBS inhibitors (39). The primary function of CBS is to covert homocysteine and serine to form cystathionine, which is a pseudo-C_2 symmetric molecule. It is known that cystathionine possesses much higher binding recognition toward CBS than other substrates, including serine, cysteine, and homocysteine (Michaelis constant [K_m] ~83 μM for cystathionine *vs*. m*M* values for other substrates) (3). Based on the structural and charge identity of cystathionine, a series of cystathionine analogs with vicinal heteroatomic groups (-$NHNH_2$, -ONH_2, -NHOH) replacing the two α-NH_2 groups were prepared and tested. Among these analogs, the hydrazine compound **6S** was found to be most promising. Ultraviolet/visible spectroscopy demonstrated that **6S** was able to engage the PLP cofactor in a stable hydrazone adduct. In both *in vitro* kinetic assays and brain whole-cell lysates **6S** showed good activity in attenuating H_2S production (inhibition constant [K_i] ~50 μM). In an *in vivo* rat stroke model (transient middle cerebral artery occlusion), **6S** showed significant activity in decreasing infarction volume. The administration of **6S** (1.6 μmol/kg, intracerebroventricular injection) led to 83% or 66% reduction in infraction volume.

High-throughput screening identified CBS inhibitors. High-throughput screening has been used in finding CBS inhibitors. Barrios and colleagues used a H_2S fluorescent probe 7-azido-4-methylcoumarin (AzMC) for this purpose (66). This probe could sense the production of H_2S and provided H_2S-concentration-dependent fluorescence readouts. It was used to evaluate the activity of CBS (or CSE), as well as the inhibitory efficacy of inhibitors. Using this method, 12 of 1900 compounds were initially found to be potent (with significant inhibition at 150 μM) for CBS. However, some of them were later found to be false positives due to two possible reasons: (i) some compounds reacted with H_2S directly and led to low fluorescence responses; (ii) some compounds quenched the fluorophore (coumarin) and weakened fluorescent signals. In addition, the candidates' selectivity for CSE was tested. Eventually, tangeritin and 1,4-naphthoquinone (Fig. 5) were found to be relatively selective and potent for CBS.

In 2013, Wu and coworkers reported a tandem-microwell-based assay for measuring CBS/CSE-mediated H_2S production (84). This method was used to screen 21,599 compounds, and several potential CBS inhibitors with IC_{50} values in low two-digit μM range were identified. For example, NSC111041 and NSC67078 were found to be most potent for CBS (IC_{50} = 4 and 12 μM, respectively). However, their selectivity was less satisfactory (IC_{50} for CSE were 2.5 and 30 μM, respectively). Quinaldine blue and MBS08407 were less potent for CBS (IC_{50} = 20 and 25 μM, respectively) but more selective as they hardly affected CSE. A follow-up study by the same group identified a more potent and selective CBS inhibitor—CH004 (71). The IC_{50} of CH004 for CBS was ~1 μM, which was ~30-fold stronger than that for CSE. As for the mode of action, it was found that CH004 binds reversibly to CBS. In kinetic studies, CH004 showed noncompetitive inhibition toward PLP, and mixed-type inhibition toward Cys and Hcys. Surface plasmon resonance studies showed that CH004 binds to CBS with a K_D value of 0.6 μM. CH004 was used to understand the links between liver cancer and CBS. It was found that CH004-mediated CBS inhibition could lead to ferroptotic cancer death in cell models and effective reduction of tumor growth in a liver tumor xenograft mice model.

Another known reaction of CBS is that it can catalyze the transformation of methylcysteine to produce serine and methanethiol (42). The production of methanethiol can be continuously monitored by 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM), a commercial fluorescent thiol probe. This was used as a high-throughput method to identify CBS inhibitors by Niu *et al.* (41). A natural product library with 6491 compounds was screened, and 11 compounds with IC_{50} <20 μM were selected. Among these hits, hypericin was most potent with IC_{50} of 3 μM. Hypericin also

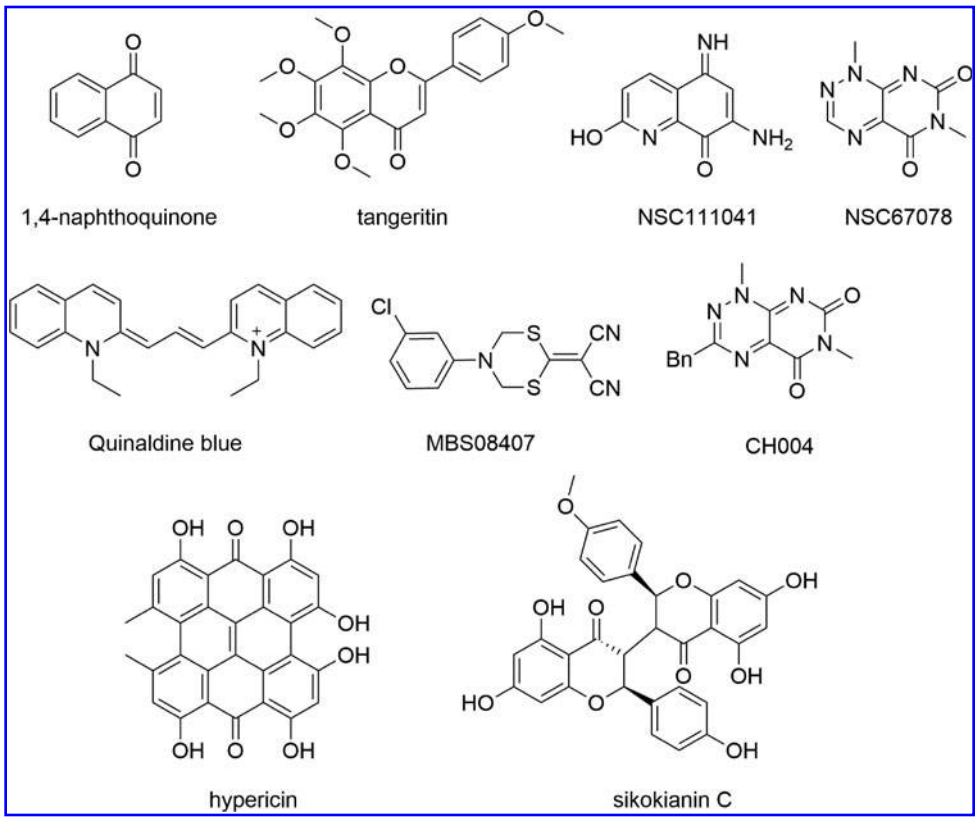

FIG. 5. High-throughput screening identified CBS inhibitors.

showed >10-fold selectivity for CBS over CSE. Another hit, sikokianin C, showed higher IC_{50} for CBS (9 μM) but better selectivity (IC_{50} >400 μM for CSE) than hypericin.

CSE inhibitors

In 1973, Abeles and Walsh reported PAG as an irreversible CSE inhibitor (2). Different from most PLP-dependent enzyme inhibitors, the mechanism of PAG inhibition is controlled by several key amino acid residues of CSE, including Arg^{62}, Lys^{212}, and Tyr^{114} (61). As shown in Figure 6, the α-amino group of PAG is first deprotonated by Arg^{62} to create the activated species, which then undergoes transaldimination to form intermediate **5**. Lys^{212} next abstracts a proton from the alkyne to form an activated allene **6**. Finally, the hydroxyl group of Tyr^{114} reacts with allene to produce vinylether **7**. The internal aldimine can be regenerated by subsequent transaldimination with Lys^{212}. This process irreversibly modifies Tyr^{114} in the active center of CSE, and therefore, deactivates the enzyme function. Although PAG is a commonly used CSE inhibitor, it has two limitations: (i) PAG is not a very potent inhibitor with IC_{50} of 40 μM (5). In living cells, much higher PAG concentrations (1–10 mM) are needed to block H_2S generation. (ii) In addition to CSE, PAG acts on other enzymes. For example, Morino and colleagues demonstrated that PAG decreased the activity of alanine aminotransferase, because increased alanine level was found in urine of PAG-treated mice (60). Compared with PAG, β-cyano-$_L$-alanine (BCA) and $_L$-aminoethoxyvinylglycine (AVG) are more potent CSE inhibitors with IC_{50} of 14 and 1 μM, respectively (5, 50). In cell- and tissue-based studies, the treatment with BCA inhibits H_2S generation in a concentration-dependent manner, while data illustrating biological function of AVG at cellular or tissue level are still lacking (64). Both BCA and AVG suffer selectivity issues. For example, BCA is potentially neurotoxic, and AVG inhibits other PLP-dependent enzymes, such as cystathionine-β-lyase (CBL) (16, 57).

Inspired by the inhibitory mechanism of PAG, a series of compounds, which fused the propargyl group with modified cysteine structures, were prepared and tested by Caliendo and colleagues (17). Among these compounds, oxothiazolidine derivative **8** showed the maximum inhibitory effect on purified CSE without affecting CBS. Compared with PAG, the IC_{50} of **8** was improved by 100-fold at tissue level. In addition, the inhibitory effect of **8** on CSE is more comprehensive. The reactions catalyzed by CSE are not only limited to converting $_L$-cysteine to pyruvate, ammonia, and H_2S, but also include converting $_L$-cysteine to lanthionine. **8** induced the inhibition of both functions, whereas PAG failed to block CSE-promoted lanthionine generation. The inhibition of CSE by **8** involves a competitive mechanism, while PAG is an irreversible inhibitor.

D-Penicillamine was used as a drug for ameliorating rheumatic disease. Taking advantage of its structural similarity to cysteine (a CSE substrate), Cirino and colleagues applied *D*-penicillamine as a CSE inhibiter in 2016 (10). After treating CSE with *D*-penicillamine, reduced H_2S generation was observed in cell-free assay. *D*-Penicillamine was found to be relatively selective toward CSE. The activity of *D*-penicillamine toward CSE (IC_{50} = 0.27 mM) was ~31-fold stronger than that for CBS (IC_{50} = 8.5 mM). PLP was believed to be the reacting site of *D*-penicillamine as the addition of extra PLP reversed *D*-penicillamine-induced

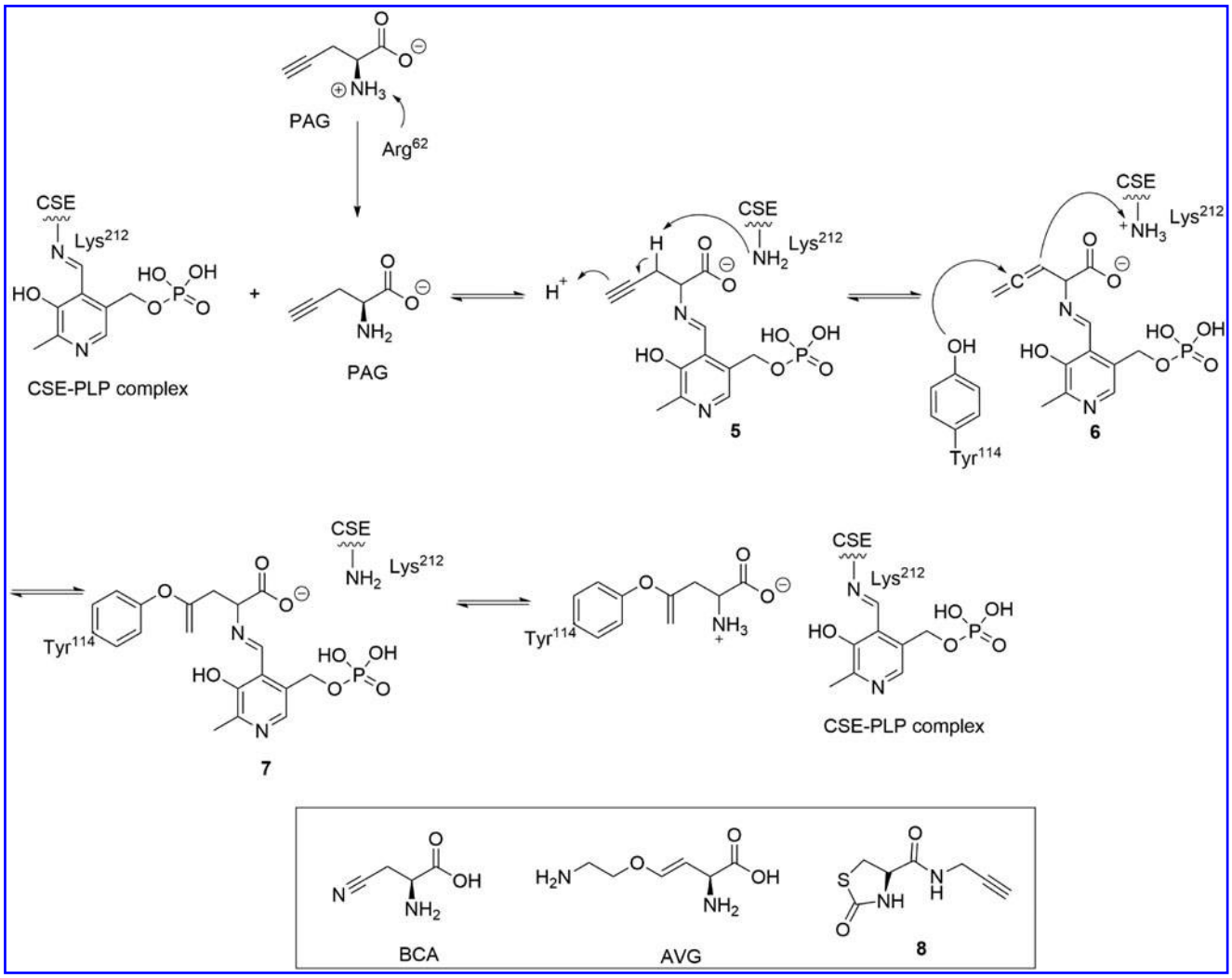

FIG. 6. Inhibitory mechanism of PAG and PAG-derived CSE inhibitors. CSE, cystathionine-γ-lyase; PAG, propargylglycine.

inhibition. The activity of *D*-penicillamine was also confirmed by *in vitro* experiments. For example, the treatment of *D*-penicillamine decreased H_2S generation and exacerbated vascular inflammation in a mouse model.

High-throughput screening was also used in the discovery of CSE inhibitors. Wardrop and colleagues screened >100,000 compounds against recombinant human CSE and identified Schiff base **9** (Fig. 7) as a hit (8). They further prepared 32 analogs and studied the structure–activity relationships (SARs). It was found that the 2-pyridyl group (R_1) and the hydrogen atom (R_2) on **9** were essential for maintaining high inhibitory efficacy on CSE, while the thioester substituent could be modified. This work led to the discovery of compound **10** as the most potent and selective CSE

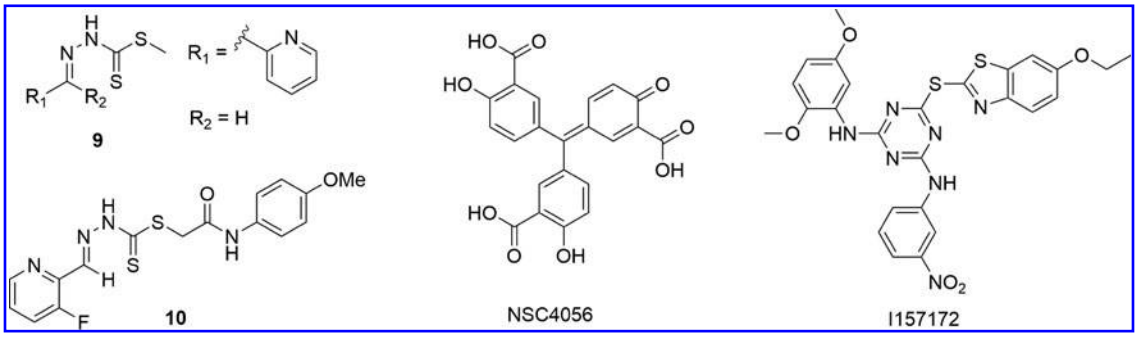

FIG. 7. High-throughput screening identified CSE inhibitors.

inhibitor. Its IC_{50} for CSE was 1.2 μM, ~400-fold lower than that for CBS. Nevertheless, the application of **10** in cell- or animal-based studies is still lacking.

Wu and coworkers developed a tandem-microwell-based assay that can constantly measure enzyme-catalyzed H_2S generation (84). This method was first used in the identification of CBS inhibitors. Very recently, it was also used in CSE inhibitor discovery (29). Eleven thousand nine hundred fifty-four compounds were screened against hCSE, and one potent hit-NSC4056 was identified. In buffers, dose-dependent CSE inhibition was induced by NSC4056, with an IC_{50} of 0.6 μM. This compound also showed high specificity for CSE. The IC_{50} for two other PLP-dependent enzymes, CBS and DOPA decarboxylase (DDC), were 137- and 143-fold higher than those for CSE. Counterscreen assay demonstrated that the effect of NSC4056 on H_2S reduction was not due to direct absorption. The study of SAR revealed that the carboxyl groups and tripod-like moiety of NSC4056 were essential. In Raw264.7 cells NSC4056 was found to reduce endogenous H_2S levels in a dose-dependent manner with an IC_{50} of 43.2 μM. It also effectively relieved hypotension in hemorrhagic shock rats.

Wang *et al.* used a virtual screening technique to discover CSE inhibitors (72). A SPECS compound library (~200,000 compounds) was created and used to study their binding affinity toward CSE. High-throughput rigid docking followed by flexible docking with force-field refinement was used to rank the compounds. I157172 was identified as the top hit with the highest binding affinity. Methylene blue assay showed that I157172 significantly decreased H_2S generation in MCF7 breast cancer cells. Interestingly, Western blot analysis also revealed that I157172 inhibited CSE expression in MCF7 cells. Eventually I157172 was found to effectively inhibit the proliferation, migration, and invasion of MCF7 cells. Mechanistic studies suggested that the effects of I157172 were linked to the sirtuin 1 (SIRT1)/acetyl-STAT3 (signal transducer and activator of transcription 3) signaling pathway. In human breast cancer cells and tissues, the expressions of CSE and SIRT1 were negatively associated. I157172-triggered CSE inhibition would promote SIRT1-mediated deacetylation of STAT3 in breast cancer cells and consequently inhibit the growth of breast cancer cells.

3-MST inhibitors

In biological systems, 3-MST not only catalyzes H_2S generation but also promotes the conversion of cyanide to thiocyanate. Wing and Baskin tested a series of compounds as potential inhibitors by measuring their effects on the rate of thiocyanate formation (77). Phenylpyruvate showed the highest activity. Under the treatment of 30 mM phenylpyruvate, the enzyme's activity dropped by 99.8% in bovine kidney extract or by 89% with purified 3-MST. 3-MP is known as the substrate of 3-MST. Two 3-MP derivatives, 3-mercaptopropionic acid (3-MPA) and 2-mercaptopropionic acid (2-MPA), were also tested (52). Both were found to be weak inhibitors (while IC_{50} data were not provided) but with different binding mechanisms. 3-MPA can bind to both free and substrate-complexed enzymes as a noncompetitive inhibitor. 2-MPA can only bind to the latter, therefore being an uncompetitive inhibitor. These studies provided a good starting point for characterizing 3-MST functions. However, their application as 3-MST inhibitors is limited due to their low potency and unclear specificity for 3-MST.

To identify more effective and selective 3-MST inhibitors, Hanaoka *et al.* screened 174,118 compounds using HSip-1 (a H_2S fluorescent probe) assay and identified a lead compound **11** (Fig. 8) (25). **11** inhibited 3-MST-catalyzed H_2S generation with an IC_{50} of 2.7 μM. **11** was also found to be selective for 3-MST as it was almost inactive for CBS, CSE, and rhodanese. When 1 μM **11** was applied to 3-MST-overexpressed COS7 cells, the activity of 3-MST was completely suppressed, demonstrating **11** as a cell-permeable inhibitor. Theoretical calculations were used to understand the interaction between **11** and the enzyme. The target of **11** was believed to be the persulfidated cysteine residue in the active site of 3-MST. A long-range electrostatic interaction between positively charged carbonyl carbon of pyrimidone moiety in **11** and persulfidated cysteine anion in 3-MST was identified as the main factor. **11**should be a useful tool in 3-MST research. For example, Panagaki *et al.* used **11** to reveal biological roles of 3-MST and H_2S in endoplasmic reticulum (ER) stress (48). NaHS treatment mitigated aspects of ER-stress, while the inhibition or silencing of 3-MST exacerbated ER-stress-related readouts.

H_2S scavengers

As H_2S-producing enzymes are ubiquitously present and involved in complex sulfur metabolisms, interfering with the activity of these enzymes may lead to unwanted side effects, and this is a general concern of the inhibitors. An alternative approach is to develop small-molecule H_2S scavengers. Ideally the scavengers should specifically and rapidly remove H_2S, therefore, only eliminating the functions of H_2S from certain biological systems. By doing so, interference with other necessary enzyme activities unrelated to H_2S could be avoided. Another advantage of using scavengers is that they can be delivered to specific cellular locations when conjugated with certain targeting moieties. As such, this would allow targeted H_2S clearance. So far, reports on H_2S scavengers have been very limited and are summarized below.

Hydroxocobalamin. H_2S is also known as a highly toxic species due to its environmental and industrial exposure. Its toxic mechanism is believed to inhibit mitochondrial

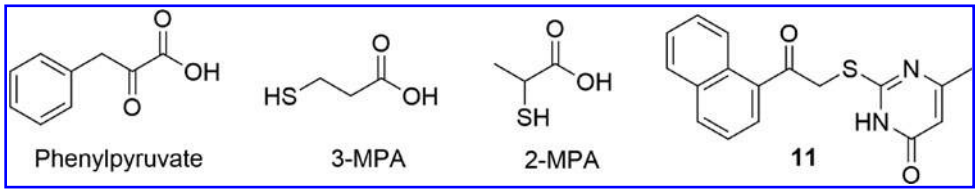

FIG. 8. 3-MST inhibitors. 3-MST, 3-mercaptopyruvate sulfur transferase.

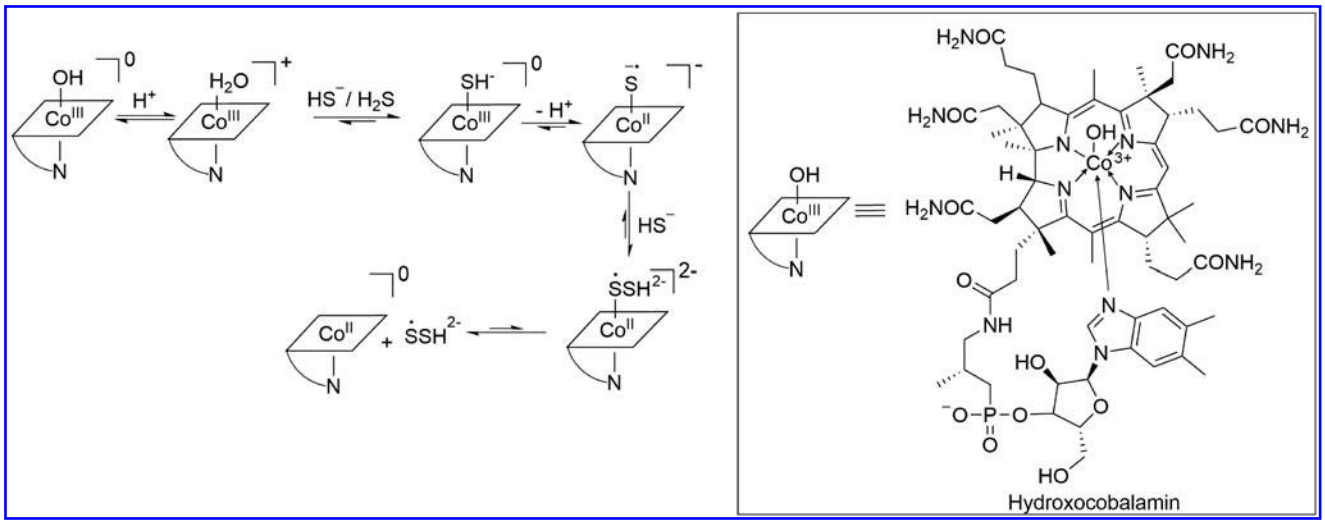

FIG. 9. The reaction between HC and H_2S. HC, hydroxocobalamin.

respiration by complexing cytochrome c oxidase copper and/or heme iron site, as well as by inducing hepatocyte reactive oxygen species (ROS) formation. Hydroxocobalamin (HC; *e.g.*, vitamin B_{12a}) has been investigated as a potential antidote for H_2S poisoning. As such, HC may be considered as a potential H_2S scavenger. In the report by Truong *et al.,* HC was found to be effective at preventing NaSH-induced mice death at all concentrations tested (67). In a cell-free system, HC increased the rate of oxygen consumption induced by NaSH, which was prevented by catalase. Catalytic concentrations of HC (25–150 μM) depleted ~95% NaSH from the original concentration (500 μM) in 1 h. A more recent work showed that within 10 min of mixing an equimolar concentration of HC and NaHS (100 μM), the sulfide concentration dropped by 95% (68). HC has been suggested as a useful H_2S-scavenging tool for studying H_2S functions in cells. In a report by Obeso and colleagues, HC was used to study the involvement of endogenous H_2S in carotid body (CB) oxygen sensing (23). It was found that a H_2S donor GYY4137 elicited catecholamine release from the whole carotid bodies with HC preventing this response. HC also abolished the rise of $[Ca^{2+}]$ evoked by NaHS in enzymatically dispersed CB glomus cells. Salnikov *et al.* carried out a detailed kinetic study of the reaction between HC and H_2S in water (pH 1–10) (58). Their suggested reaction mechanism is shown in Figure 9. H_2S or HS^- first forms a complex with aquacobalamin (the conjugate acid of hydroxocobalamin). This then promotes inner-sphere electron transfer to oxidize H_2S to $S^{-\bullet}$ and reduce the cobalt(III) ion (Co^{3+}) in the HC core to cobalt(II) ion (Co^{2+}). Finally, the addition of another molecule of HS^- to the bound $S^{-\bullet}$ produces $SSH^{2-\bullet}$, which is released as anion radical $SSH_2^{-\bullet}$ in acidic medium. This unstable species eventually should degrade to form elemental sulfur. The problem of HC as a H_2S scavenger is its poor specificity. In addition to H_2S, HC scavenges cyanide, NO, CO, and ROS (11, 31, 67). This nonspecificity may be fine for HC as an antidote for H_2S poisoning but should limit its use as a selective H_2S scavenger. Nevertheless, NO, CO, or even some ROS are known as signaling molecules. Removing all of these could cause unexpected consequences.

Sulfonyl azides. Our laboratory explored small-molecule compounds as H_2S scavengers (79). We envisioned the following criteria for ''ideal'' scavengers: (i) the scavengers should possess high reactivity to H_2S. The reaction should be completed within minutes, not in hours. (ii) The scavengers should be highly selective to H_2S. They should only react with H_2S, not with other cellular species. (iii) The scavengers and their H_2S reaction products should possess minimum biological activity. We recognized that these criteria are very much the same as the criteria for H_2S sensors. H_2S sensors have been extensively studied, and hundreds of such sensors have been reported. Therefore, we built up a comprehensive H_2S sensor database and analyzed their reactions with H_2S. We focused on the time needed for the sensors to complete the reaction with H_2S and the specific H_2S-reactive functional groups. This data-driven approach allowed us to identify possible chemical entities that could react with H_2S rapidly and specifically. We then prepared a number of such compounds, and tested their H_2S-scavenging ability and specificity. A Unisense H_2S microsensor (Tueager 1, Denmark) was used in these studies. Eventually, a series of

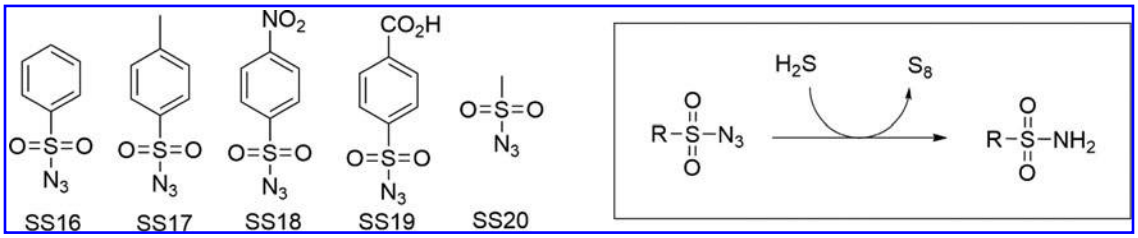

FIG. 10. Sulfonyl azide-based H_2S scavengers.

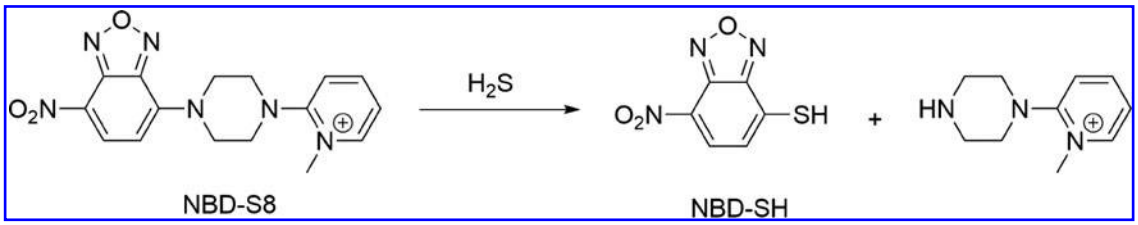

FIG. 11. NBD amine-based H_2S scavenger. NBD, 7-nitro-1,2,3-benzoxadiazole.

sulfonyl azides (Fig. 10) were found to be potent scavengers. These compounds could remove H_2S from buffers in a few minutes, and the presence of biothiols (Cys or GSH) did not seem to interfere with their scavenging ability. One scavenger SS19 showed dose-dependent H_2S removal in an enzymatic sulfide generation system (using EcCARS). Another scavenger SS17 was tested in cell models to mitigate the effects of H_2S. For example, the pretreatment with SS17 for 30 min before H_2S exposure markedly attenuated the effects of H_2S on cell growth in SNU398 cells. However, it should be noted that the pretreatment with scavengers in cell models is not an ideal way for the evaluation of scavengers. To better mimic the actual scenarios, scavengers should be applied after H_2S overproduction is induced. In a mouse model of H_2S intoxication, SS20 showed excellent activity as the antidote at 250 μmol/kg *via* intraperitoneal injection. SS20 was also used by Olson *et al.* in removing endogenously formed H_2S, which served as part of the evidence to show lipoic acid could concentration dependently induce H_2S production (45). The reaction between H_2S and sulfonyl azides is shown in Figure 10. It is a redox reaction, similar to the reaction between H_2S and aryl-azides (27). Sulfonyl azides are converted to sulfonamides, and H_2S is converted to S_8. In this process, hydrogen polysulfides (H_2S_n) may be formed as the intermediates. However, H_2S_n should also react rapidly with sulfonyl azides to form S_8.

7-Nitro-1,2,3-benzoxadiazole amines. Ismail *et al.* reported a series of 7-nitro-1,2,3-benzoxadiazole (NBD) amines as potential H_2S scavengers (30). These compounds are based on NBD-type H_2S fluorescent sensors. It was found that these compounds could specifically react with H_2S *via* thiolysis to form NBD-SH and the corresponding amines (Fig. 11). The reaction kinetics are dependent on the structures of amines. One compound, NBD-S8, showed the highest reactivity toward H_2S ($k_2 = 116.1\ M^{-1} \cdot s^{-1}$). It also showed good stability in buffers and high specificity for H_2S over biothiols. H_2S-scavenging ability of NBD-S8 was further evaluated in 10% fetal bovine serum, cells (HeLa and FHC), and mice, by monitoring H_2S concentration changes in these systems with fluorescence measurements. Overall, NBD-S8 showed satisfactory results, demonstrating that it is a promising scavenger.

Conclusions

H_2S has been classified as an important signaling molecule, and dysregulation of H_2S is linked to a number of pathological processes, including cancer, inflammation, and neurodegenerative diseases. Regulation of H_2S levels is believed to have therapeutic potential, and this requires chemical compounds that can precisely regulate local H_2S concentrations. This is still a challenging topic as the therapeutic window of H_2S is very narrow. In the past decade, many inhibitors of H_2S-producing enzymes have been reported, and recently H_2S scavengers started to attract some attention. Nevertheless, there are still limitations with these chemicals. The general concerns regarding inhibitors are their specificity and possible side effects. H_2S-producing enzymes are present ubiquitously in biological matrices and involved in complex sulfur metabolic pathways. The blockage of the metabolic pathways catalyzed by these enzymes thereby may affect the downstream cysteine production rather than H_2S *per se*. Any pharmacological consequences obtained by the inhibitors can not to be solely attributed to the reduction of H_2S biosynthesis. This mechanism-based problem seems to be unavoidable even for the most selective inhibitors. Scavengers have the potential to remove H_2S in certain specific tissue or cellular locations. However, up to now, the options of specific H_2S scavengers have been very limited. In addition, the use of scavengers would generate H_2S-derived reaction products, which could cause unexpected side effects. Some precautions should be kept in mind when using scavengers in research. For example, the sulfonyl azide-based scavengers would produce sulfonamides and S_8 when reacting with H_2S. Control experiments with the corresponding sulfonamide and S_8 should be performed to rule out the effects from these by-products. Another problem is that current H_2S scavengers are based on stoichiometric reactions with H_2S. Therefore, high doses of scavengers are needed if H_2S is continuously generated. This problem may be solved if catalytic H_2S scavengers are developed, but this has yet to be explored. We expect to see more research on H_2S-suppressing methods in the coming years.

Authors' Contributions

Y.W. and M.X. conceived the presented review. Y.W., X.N., R.C., C.M., Y.L., B.B., G.R., and M.X. contributed to the writing of the article.

Author Disclosure Statement

There are no conflicts to disclose.

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Abbreviations Used

2-MPA = 2-mercaptopropionic acid
3-MP = 3-mercaptopyruvate
3-MPA = 3-mercaptopropionic acid
3-MST = 3-mercaptopyruvate sulfur transferase
AOAA = aminooxyacetic acid
Arg = arginine
AVG = $_L$-aminoethoxyvinylglycine
BCA = β-cyano-$_L$-alanine
Ca^{2+} = calcium ion
CARS = cysteinyl-tRNA synthetases
CAT = cysteine aminotransferase
CB = carotid body
CBS = cystathionine-β-synthase
CO = carbon monoxide

Abbreviations Used (Cont.)

CSE = cystathionine-γ-lyase
DSCs = Down syndrome cells
ER = endoplasmic reticulum
GSH = glutathione
H_2S = hydrogen sulfide
H_2S_n = hydrogen polysulfide
HC = hydroxocobalamin
IC_{50} = half-maximal inhibitory concentration
Lys = lysine
NADPH = nicotinamide adenine dinucleotide phosphate
NBD = 7-nitro-1,2,3-benzoxadiazole
NH_3 = ammonia
NO = nitric oxide
PAG = propargylglycine
PLP = pyridoxal-5′-phosphate
ROS = reactive oxygen species
S_8 = elemental sulfur
SARs = structure–activity relationships
SIRT1 = sirtuin 1
STAT3 = signal transducer and activator of transcription 3
THBH = 2,3,4-trihydroxylbenzylhydrazine
Tyr = tyrosine