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Hydrogen sulfide prodrugs—a review

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Abstract  Hydrogen sulfide (H\textsubscript{2}S) is recognized as one of three gasotransmitters together with nitric oxide (NO) and carbon monoxide (CO). As a signaling molecule, H\textsubscript{2}S plays an important role in physiology and shows great potential in pharmaceutical applications. Along this line, there is a need for the development of H\textsubscript{2}S prodrugs for various reasons. In this review, we summarize different H\textsubscript{2}S prodrugs, their chemical properties, and some of their potential therapeutic applications.
1. Introduction

Hydrogen sulfide (H₂S), a well-known lethal, toxic gas with the smell of rotten eggs, is recognized as one of the three gasotransmitters in mammals, which also include nitric oxide (NO) and carbon monoxide (CO)\(^\text{19-24}\). The literature evidence suggests that hydrogen sulfide possesses the following activities: anti-inflammatory\(^\text{7-8}\), anti-tumor\(^\text{10}\), ion channel regulation\(^\text{11-13}\), cardiovascular protection\(^\text{14-16}\) and antioxidation\(^\text{17}\). However, the exact role that hydrogen sulfide plays depends on the specific circumstance, its concentration, and the interplays with other signaling molecules, especially NO and CO\(^\text{18}\). This is a major area of research in developing hydrogen sulfide-based therapeutics, but is beyond the scope of this review. Another major issue is finding appropriate ways of delivering hydrogen sulfide to the relevant location, at the right concentration, and with the appropriate pharmacokinetics. Much of this issue stems from the fact that it is unrealistic to use gaseous hydrogen sulfide itself or its salt such as sodium sulfide in therapeutic applications in human. Thus there is a great deal of interest in searching for appropriate hydrogen-sulfide-releasing agents, which are commonly referred to as H₂S donors or prodrugs. This review provides a summary of developments in this field mostly during the last five years with a focus on the chemistry concepts\(^\text{19}\).

1.1. H₂S chemistry

H₂S is a weak acid and soluble in water (up to 80 mmol/L at 37 °C\(^\text{25}\)). The \(pK_a\) values (37 °C) for the first and second dissociation steps are about 6.88 and 19, respectively\(^\text{21}\). Under physiological conditions (pH≈7.4), H₂S largely exists in two forms: the neutral molecular form (H₂S) and an ionic form (H\(^\text{S}^-\)) (Scheme 1). S\(^\text{2-}\) is a very minor component simply because of the second \(pK_a\) being very high. However, the bioactive form is still unknown, and the term H₂S is usually used referring to the total sulfide species. Although H₂S has good solubility in water, it is still very unstable in solution. It is easy oxidized in the presence of oxygen. In addition, the volatility of hydrogen sulfide adds complications to experiments. For example, half of the dose of H₂S could be lost in 5 min from open cell culture wells\(^\text{22}\). H₂S concentration can decrease so rapidly that the precise measurement of H₂S concentration is a great challenge in this field\(^\text{23-28}\).

1.2. H₂S biology

In mammals, three enzymes are involved in sulfur-containing amino acid metabolism and thus responsible for the \textit{in vivo} production of H₂S. Two of them are pyridoxal-5’-phosphate (PLP)-dependent enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). CBS is expressed predominantly in the central nervous system (CNS)\(^\text{29}\). Relatively high concentrations (47 μmol/L to 166 μmol/L) of H₂S have been observed in the brains of mammals\(^\text{30-32}\). The normal cellular function of CBS is in the trans-sulfuration pathway, catalyzing the condensation of homocysteine with serine to form cystathionine. In the 1980s, CBS isolated from rat liver and kidney was reported to produce H₂S from cysteine\(^\text{33}\). In contrast to CBS, CSE is mainly responsible for the production of H₂S outside of the CNS\(^\text{2}\). CBS and CSE share a common feature of catalytic promiscuity. The relative contributions of CBS and CSE to H₂S generation at low homocysteine concentration are about 7:3. However, CBS activity is confined to chemical transformations at the β-position\(^\text{34}\), while CSE is proficient at catalyzing reactions at the β- and γ-carbons of substrates\(^\text{35}\). Furthermore, because homocysteine appears to be unable to bind to the site at which the external aldimine with PLP is formed in CBS, CSE's contribution to the H₂S pool is increased under conditions of moderate and severe hyperhomocysteinemia. A third H₂S-producing enzyme, 3-mercaptopyruvate sulfurtransferase (3MST), was thought to exist, as H₂S was not depleted in CBS knockout mouse brain\(^\text{36}\). 3MST, a PLP-independent enzyme, is localized in the neurons in the brain along with cysteine aminotransferase (CAT), while CBS is localized in the astrocytes, a type of glia, in the CNS. 3MST and CAT are also found in the vascular endothelium. CAT catalyzes the reaction of l-cysteine with α-ketoglutarate to form 3-mercaptoppyruvate (3MP), which is further catalyzed by 3MST to generate H₂S in the presence of thiol and reducing agents (Fig. 1)\(^\text{37}\). Overall, H₂S production in mammals is intimately connected to the metabolic pathways of sulfur containing amino acids. The PLP-dependent trans-sulfuration pathway, which contains both CBS and CSE for H₂S production, is localized in the cytosol, H₂S synthesis \textit{via} CAT and 3MST occurs in the cytosol and mitochondria.

![Figure 1](image-url) Enzymatic pathways of H₂S production in mammalian cells.
vassaultivity of garlic compounds was correlated with H₂S production, which suggested that the major beneficial effects of allium vegetable diets are mediated by the biological production of H₂S from organic polysulfides.

To date, several sulfur-containing components from garlic or garlic preparations have been identified (γ-glutamylcysteines and allicin in the intact garlic; ajoene and allyl mercaptan in the steam-distilled garlic oil; S-allyl-cysteine and S-allyl-mercaptopcysteine in the aged garlic extract; and methiin in the garlic homogenate). Among all the different components, only three of them, S-allyl-cystein (SAC), diallyl disulfide (DADS), and diallyl trisulfide (DATS) have been shown to have pharmacological effects, which are correlated with the H₂S signaling pathway. DADS and DATS are major components of garlic oil, and are derived from allicin, which is unstable in H₂S from DADS.

The third sulfur-containing compound in allium vegetables related to H₂S production is S-allyl-cysteine (SAC), a reduced form of alliin, which is the major component in aged garlic extract. Studies from Zhu et al. showed that SAC and CR-SPRC, a cysteine analog of SAC, upregulated CSE expression and plasma H₂S concentrations. Rats used in an acute myocardial infarction and heart failure model were treated with SAC or CR-SPRC, respectively. It was found that SAC and its analog significantly lowered mortality and improved cardiac function. The activity of CSE, CAT, GSH, and plasma H₂S concentration were increased in SAC-pretreated and CR-SPRC-treated rats, suggesting its cardioprotection effect via a H₂S-mediated pathway. However, there is no report on H₂S production directly from SAC in the biological systems.

Recently, Kondo et al. published a H₂S prodrugs: SG-1002 (Fig. 2), which is a polysulfur mixture containing 92% α sulfur, 7% sodium sulfate and a trace amount of other sulfur derivatives. In one study, SG-1002 was administered to C57BL/6J or CSE knockout mice to investigate the effects of genetic modulation of CSE and exogenous H₂S in a pressure overload-induced heart failure model. It was found that CSE knockout mice exhibited significantly greater cardiac dilatation and dysfunction than wild-type mice after transverse aortic constriction, and cardiac-specific CSE transgenic mice maintained cardiac structure and function after transverse aortic constriction. H₂S afforded by SG-1002 could upregulate the vascular endothelial growth factor (VEGF)-Akt-endothelial nitric oxide synthase (eNOS)-nitric oxide (NO)-cGMP pathway with preserved mitochondrial functions, attenuated oxidative stress, and increased myocardial vascular density. The results show oral H₂S therapy prevents the transition from compensated to decompensated heart failure in part via upregulation of endothelial nitric oxide synthase and increased nitric oxide bioavailability. However what needs to be noted concerning these studies is that the mechanism of H₂S release from SG-1002 is not described. More studies are needed to prove the correlation of H₂S production and the observed pharmacological effects.

2.2. Hydrolysis-based H₂S prodrugs

Hydrolysis-based H₂S prodrugs primarily consist of four classes of analogs: namely, inorganic sulfite salts including NaHS, Na₂S and CaS; Lawesson’s reagent and analogs; 1,2-dithiole-3-thiones; and arylthioamides derivatives. For arylthioamides derivatives, some classified them as thiol-activated H₂S donors. Since these compounds are easily hydrolyzed to generate H₂S in PBS buffer, they are summarized in this section.

2.2.1. Inorganic sulfite salts
NaHS and Na₂S are two widely used H₂S donors in basic research. Upon hydrolysis, both compounds could generate H₂S quickly in PBS buffer (pH 7.4). In aqueous state under physiological pH, the ratio of HS⁻/H₂S is around 3:1. NaHS treatment could increase peak expiratory flow (PEF), and decrease goblet cell hyperplasia, collagen deposition score, the total cells recovered from bronchoalveolar fluid, and influx of eosinophils and neutrophils. Additionally, administration of NaHS also significantly attenuated activation of pulmonary inducible nitric oxide synthase (iNOS). Those results suggested that H₂S possessed anti-inflammatory and anti-fibrotic effect.
anti-remodeling effect in asthma pathogenesis, presumably by the cystathionine-gamma-lyase (CSE)/H₂S pathway. Using NaHS as a H₂S donor, Du et al. examined the possible role of H₂S in the pathogenesis of oleic acid (OA)-induced acute lung injury (ALI) and its regulatory effects on the inflammatory response. Intraperitoneal injection of NaHS (56 μmol/L) into OA-treated rats increased the pressure of oxygen in the arterial blood (PaO₂), reduced the lung wet/dry ratio and alleviated the degree of ALI. Additionally, NaHS decreased inflammatory cytokine such as IL-1β and IL-8 levels and increased anti-inflammatory cytokine IL-10 levels in the plasma and lung tissues. Similarly, Na₂S inhibited IL-1β levels and significantly increased anti-inflammatory cytokine IL-10 levels in an acute lung injury model.

In addition to anti-inflammatory effect, NaHS or Na₂S also showed pro-inflammatory effects, ion channel regulation, cardiovascular, and neurogenic regulation effects. Although NaHS and Na₂S presented promising results both in vitro and in vivo, the likelihood of their use in clinical applications is small due to reasons such as release kinetics, smell, lack of ability to target, and difficulty in controlling its concentration because of hydrogen sulfide's volatility. Nevertheless, encouragingly, a sodium sulfide solution (IK-1001) for intravenous injection has successfully completed a phase I clinical trial, thus pointing to the possibility of applications in well-defined situations.

Another potential inorganic H₂S donor is calcium sulfide (CaS), which is one of the effective components in a traditional medicine, hep ar sulphuris calcarea. Compared to NaHS and Na₂S, CaS is chemically more stable. However, there is only limited information on the effectiveness of CaS as H₂S donor.

2.2.2. Lawesson's reagent and analogs
Lawesson's reagent, which is widely used for sulfuration in organic synthesis, also releases H₂S upon hydrolysis, and it has been used as a H₂S donor in some studies. Compared to inorganic sulfide, the release rate with Lawesson's reagent is much slower. After incubation of Lawesson's reagent in buffer or rat liver homogenate for 60 min, the conversion to H₂S was about 18% or 11%, respectively. In work by Medeiro et al., Lawesson's reagent was used as H₂S donor to evaluate its protective effect on the effectiveness of CaS as H₂S donor.

GYY4137, a water-soluble derivative of Lawesson's reagent, could also release H₂S upon hydrolysis. Compared to Lawesson's reagent and sulfide salts, it can generate H₂S at a slower rate. With the use of the H₂S microelectrode or the DTNB (5,5-dithiobis-(2-nitrobenzoic acid)) assay, it was found that incubation of GYY4137 in aqueous solution (pH 7.4, 37 °C) resulted in the release H₂S, and the concentration of H₂S peaked at around 6–10 min, and remained at a low level (<10 μmol/L) over a sustained period of 100 min. In one experiment, the administration of GYY4137 (133 μmol/kg i.v. or intraperitoneal) to anesthetized rats could also boost the concentration of H₂S in plasma to around 75 μmol/L at the 30 min point, and the concentration remained elevated (above 40 μmol/L) for more than 180 min. Additionally, GYY4137 did not cause any significant cytotoxic effect, or alter the cell cycle profile or p53 expression of cultured rat vascular smooth muscle cells. However, NaHS was previously reported to induce apoptotic cell death of cultured fibroblasts and smooth muscle cells. The differences in the safety profile between GYY4137 and NaHS may be attributed to the differences in H₂S release rate and the concentration of H₂S generated.

In work published by Liu et al., GYY4137 was employed as a H₂S donor to investigate its effects on CVB3-induced myocarditis and possible underlying mechanisms. The results showed that GYY4137 suppressed CVB3-induced secretion of enzymes implicated in cardiocyte damage including LDH, CK-MB, and decreased the level of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6. Moreover, GYY4137 also inhibited the activation of NFκB and the IκBα degradation induced by CVB3. Notably, the phosphorylation of p38, ERK1/2 and JNK1/2 induced by CVB3 was also suppressed by GYY4137. Taken together, GYY4137 exerted its anti-inflammatory effect in CVB3-infected cardiomyocytes, which was possibly associated with H₂S generation by GYY4137. The anti-inflammatory mechanism may be associated with the inhibition of NFκB and the mitogen-activated protein kinase (MAPK) signaling pathway.

Additionally, at a concentration of 400 or 800 μmol/L, GYY4137 also showed some anti-cancer effect with 30%–70% death in seven different human cancer cell lines (HeLa, HCT-116, Hep G2, HL-60, MCF-7, MV4-11 and U2OS) and no effect on the survival of normal human lung fibroblasts (IMR90, WI-38). In contrast, NaHS did not show any anticancer effect (400 μmol/L), and only showed less potent growth inhibition (15%–30%, 800 μmol/L). The author attributed such difference to the different H₂S release rate between GYY4137 and NaHS. Incubation of GYY4137 (400 μmol/L) in culture medium released low concentrations (<20 μmol/L) of H₂S, with the concentration sustained over a period of 7 days. In contrast, incubation of NaHS (400 μmol/L) in the same way led to much higher concentrations (up to 400 μmol/L) of H₂S with a much shorter duration (1 h). It is well-known that the effect of H₂S is concentration-dependent with high concentrations (above 250 μmol/L) being toxic, thus it is easy to understand that release kinetics and peak concentration would make much difference to the overall effect of a H₂S donor. The in vitro antitumor effect of GYY4137 was also evaluated. In a xenograft mouse model (HL-60 and MV4-11 cells), GYY4137 could significantly inhibit tumor growth at dosages of 100–300 mg/kg/day.

Despite all the success described above, other independent studies sometimes showed opposite effect when NaHS was used as a donor. For example, H₂S in the form of NaHS showed protective...
effect for colon cancer cells \textsuperscript{64}, increased proliferation of colon cancer cells, and reduced apoptosis in several cell lines \textsuperscript{65}. These disparate observations may be due to the use of different H\textsubscript{2}S donors, which release H\textsubscript{2}S at different rates, give different byproducts, and have different peak concentrations. Although ZYJ1122 (Fig. 3), an analog of GYY4137 lacking sulfur, was inactive in all cancer cell lines tested, it is unclear what byproducts GYY4137 would generate in cells, because the metabolism for GYY4137 is expected to be complicated. Additionally, it should be kept in mind that the percentage of hydrolysis for GYY4137 is low, which means that the majority of GYY4137 remained in the cells. Since relatively high concentrations of GYY4137 was used (400 and 800 \textmu mol/L), it is entirely possible that the observed anticancer effect may be caused by GYY4137 itself or its metabolism products, and not necessarily the released H\textsubscript{2}S. The convoluted situation with the observed effects of “H\textsubscript{2}S” is a strong indication that future experiments need to be benchmarked against a standard and standard conditions with careful control and documentation of concentrations.

In order to tune the \textsubscript{2}S release capability of GYY4137, structural modifications on the phosphorodithioate moiety were made to GYY4137 to afford a series of \textit{O}-substituted phosphorodithioate-based H\textsubscript{2}S donors (Fig. 3)\textsuperscript{66}. Their H\textsubscript{2}S releasing properties were evaluated by fluorescence methods. After incubation (100 \textmu mol/L) in PBS buffer (pH 7.4) for 3 h at room temperature, \textit{N,O}-diarylated donors and GYY4137 could release H\textsubscript{2}S with a final concentration of around 800 \textmu mol/mL. However, the \textit{O}-alkylated donors showed very weak H\textsubscript{2}S production (data not shown in the paper). The protective effects of \textit{N,O}-diarylated donors against H\textsubscript{2}O\textsubscript{2}-induced oxidative damage in H9C2 cells were investigated. Specifically, the donors were incubated with the cells for 24 h before H\textsubscript{2}O\textsubscript{2} was added. Then cell viability was determined by the CCK-8 assay after incubation for another 5 h. The results showed that in the absence of a H\textsubscript{2}S donor, cell viability decreased by about 65\%. In the presence of H\textsubscript{2}S donors (\textit{N,O}-diarylated donors, GYY4137 and NaHS), a much higher level cell viability was observed, especially for one \textit{N,O}-diarylated donor, which increased the cell viability to about 95\% at the concentration of 100 \textmu mol/L. These results suggested that the H\textsubscript{2}S donors did present protective effects against oxidative injury. As mentioned above, the biological results obtained for these donors should be carefully associated with the generation of H\textsubscript{2}S. Further experiments may be needed to clarify this.

2.2.3. Arylthioamides derivatives

A series of arylthioamides were synthesized by Vincenzo Calderone et al.\textsuperscript{67}, and their H\textsubscript{2}S release properties were evaluated. The synthesized compounds were incubated with or without \textit{l}-cysteine in PBS buffer at 37 °C, and H\textsubscript{2}S release was recorded by amperometry. The results showed that compounds 1-3 (Fig. 4) did not generate a detectable level of H\textsubscript{2}S (<2 \textmu mol/L) in the absence of \textit{l}-cysteine; however, they did release H\textsubscript{2}S in the presence of \textit{l}-cysteine with \textit{C}_{\text{max}} of about 10 \mumol/L. For compounds 4 and 5 with strong electron-withdrawing substituents, no detectable levels of H\textsubscript{2}S were observed with or without \textit{l}-cysteine. Based on those results, it seems that the H\textsubscript{2}S release mechanism for arylthioamides is thiol-activated, and Xian et al.\textsuperscript{68} did classify arylthioamides as thiol-activated H\textsubscript{2}S donors. However, some analogs in this series also generated detectable amounts of H\textsubscript{2}S in the absence of \textit{l}-cysteine, especially compound 12, which did not show any difference in the amount of H\textsubscript{2}S released with or without \textit{l}-cysteine. It is well characterized that hydrolysis of thioacetamide would lead to H\textsubscript{2}S formation. So it could be concluded that the hydrolysis of arylthioamides could also give H\textsubscript{2}S. Actually, Wallace et al.\textsuperscript{69} classified these compounds as hydrolysis-based H\textsubscript{2}S donors. It may be more reasonable to say that both mechanisms contribute to H\textsubscript{2}S generation because there is no clear evidence to exclude either.

After confirmation of H\textsubscript{2}S release from arylthioamides, compound 1 was chosen for further pharmacological studies. It strongly abolished the noradrenaline-induced vasoconstriction in isolated rat aortic rings and hyperpolarized the membranes of human vascular smooth muscle cells in a dose-dependent fashion. After oral administration of compound 1, the systolic blood pressure of the animals was significantly reduced. These findings make arylthioamides promising H\textsubscript{2}S donors for further study.

No matter what the H\textsubscript{2}S release mechanism for arylthioamides is, it should be noted that incubation of 1 mmol/L of the donors only release H\textsubscript{2}S with a \textit{C}_{\text{max}} value of about 10 \mumol/L, which means that the major species in solution is still the donor itself. Thus it is still premature to associate the observed bioactivities with the generation of H\textsubscript{2}S alone, because the bioactivities may be caused by the donor itself or a combination of various species. Actually, this issue is quite common among the organic H\textsubscript{2}S donors. More detailed and well-designed control experiments are needed to address this issue.

2.2.4. \textit{1,2-Dithiole-3-thiones and H\textsubscript{2}S-hybrid nonsteroidal anti-inflammatory drugs}

\textit{1,2-Dithiole-3-thiones (DTT)} has also been used as a H\textsubscript{2}S donor. Although its H\textsubscript{2}S-release mechanism is still not fully clarified, it is widely accepted that hydrolysis is part of the underlying mechanism for the generation of H\textsubscript{2}S from DTT\textsuperscript{77}. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) suffers from unacceptable risk of gastrointestinal ulceration and bleeding\textsuperscript{70-73}. In order to reduce such side effects, DTTs have been conjugated to NSAIDs to form HS-hybrid NSAIDs (HS-NSAIDs, Fig. 5), which showed significant reduction of gastrointestinal damage compared to the parent NSAIDs\textsuperscript{73,74}. In addition, HS-NSAIDs also boosted the anti-inflammatory activity of their NSAIDs counterparts. In a work by Fiorucci et al.\textsuperscript{75}, DTT was conjugated to diclofenac to afford a HS-NSAID-hybrid ATB337, and its anti-inflammatory effect was investigated along with diclofenac in rats. In a rat air pouch model, orally

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure3.png}
\caption{The chemical structures for Lawesson’s reagent-based H\textsubscript{2}S donors.}
\end{figure}
administered ATB-337 dose-dependently suppressed the activity of both COX-1 and COX-2, and the efficiency was comparable to that of the diclofenac. Additionally, pretreatment with ATB-337 and diclofenac led to a reduction of carrageenan-induced paw swelling volume. Notably, pretreatment with ATB-337 at 10 μmol/kg achieved a reduction in edema formation comparable to that seen with diclofenac at 30 μmol/kg. This enhanced potency was probably associated with the generation of H₂S from ATB-337.

An enhanced anti-inflammatory effect was also observed for ATB-429⁷⁰. In addition to their anti-inflammatory effect, other HS-NSAIDs including HS-sulindac (HS-SUL), HA-aspirin (HS-ASA), HS-ibuprofen (HS-IBU), and HS-naproxen (HS-NAP), were also reported to exhibit anti-proliferative effect against human colon, breast, pancreatic, prostate, lung, and leukemia cancer cell lines. The conjugation with 5-(4-hydroxyphenyl)-1,2-dithiol-3-thione (ADT-OH) significantly increased the growth inhibitory effect of NSAID by 28- to >3000-fold⁷⁷.

Along the line of NSAID's antiproliferation effect, Kashfi et al.⁷⁷ prepared a compound NBS-1120 (Fig. 6), which could release NO, H₂S and aspirin at the same time. NBS-1120 inhibited HT-29 colon cancer growth with IC₅₀ values of 45.5 ± 2.5, 19.7 ± 3.3, and 7.7 ± 2.2 nmol/L at 24, 48, and 72 h time points, respectively. This is the most potent NSAID-based anticancer agent so far. Mechanistic studies showed that NBS-1120 induced apoptosis, and arrested the cells at G₀/G₁ phase. NBS-1120 also showed promising in vivo antitumor effect. It significantly inhibited tumor growth by 85% in mice bearing a human colon cancer xenograft.

Accumulating evidence supports that H₂S plays a vital role in the modulation of mitochondrial cell death pathways and in the regulation of cellular bioenergetics³⁻⁴,⁸⁰. Multiple studies revealed that H₂S donors help maintain mitochondrial integrity, reduce the release of mitochondrial death signals, and attenuate mitochondrially-regulated cell death responses of various types⁷⁹,⁸¹,⁸². Szabo et al.⁸³ prepared a compound AP39 (Fig. 6) with two moieties: ADT-OH for H₂S generation and triphenylphosphinium (TPP) for mitochondrial targeting. Cell imaging studies confirmed that AP39 was primarily internalized in mitochondria.⁸⁴ After confirming the mitochondria-targeting H₂S delivery, compound AP39 was employed to investigate its effect on bioenergetics, viability, and mitochondrial DNA integrity in bEnd.3 murine microvascular endothelial cells in vitro. At a
concentration of 100 nmol/L, incubation of AP39 with bEnd.3 cells caused an increase in basal oxygen consumption rate (OCR), which represented respiratory reserve capacity, a key bioenergetic parameter. Meanwhile, AP39 (30 and 100 nmol/L) could also induce an increase in FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone)-stimulated OCR. However, when the concentration of AP39 was increased to 300 nmol/L, an inhibitory effect was observed instead. AP39 could also attenuate the loss of cellular bioenergetics during oxidative stress caused by glucose oxidase. Additionally, Co-treatment of ADT-OH with TPP targeting moiety did not show any antioxidant or cytoprotective effect in oxidatively stressed endothelial cells even at a concentration of 300 nM, which is attributed to its inability to enter mitochondrial compartment. In contrast, ADT-OH without the TPP targeting moiety did not show any antioxidant or cytoprotective effect in oxidatively stressed endothelial cells even at a concentration of 300 nmol/L, which is attributed to its inability to enter mitochondrial compartment. In summary, the various mitochondrial effects observed for AP39 are consistent with the role of H2S in the regulation of mitochondrial function.

Although DTT and its NSAID hybrids showed promising H2S-related bioactivities both in vitro and in vivo, it is still unclear how those agents release H2S in vivo. Hydrolysis for sure partially contributes to the release of H2S from DTT. Because of the presence of disulfide bonds in DTT, thiols could also activate DTT through reduction to release H2S. Therefore, further experiments are needed to elucidate its H2S release mechanism.

2.3. Controllable H2S prodrugs

The goal of controllable H2S prodrugs was to develop H2S prodrugs, which are stable in aqueous solutions and during sample preparation. The prodrugs can release H2S in the presence of triggers, which could be enzymes, pH, biomolecules, UV-light, and others. However, this is still a great challenge in this field. Currently, there are three examples: thiol activation, light activation, and bicarbonate activation.

2.3.1. Thiol activation

In 2011, Xian's group developed the first thiol activated H2S prodrugs: N-mercapto (N-SH)-based derivatives. The strategy was based on the instability of the N-SH bond. The thiol group was first protected with acyl groups, and then the protected nitrogen-sulfur bond could be stable to some degree. In the presence of thiol species in the biological system, H2S release can be triggered through reduction. A detail mechanism is shown in Scheme 4. The prodrug is first activated by thiol exchange between a thiol species (cysteine or GSH) and the prodrug to generate S-acylated cysteine and N-mercaptobenzamide. Then one of the intermediates, N-mercaptobenzamide, reacts with cysteine to form cysteine perthiol, which is followed by interaction with cysteine to release H2S. In this mechanistic study, the Xian's group found that perthiol could also be a key intermediate in H2S generation. In 2013, Xian's laboratory also tested the protective effect of these prodrugs against myocardial ischemia/reperfusion (MI/R) injury in a murine model system, since H2S was proven to show such effects. In these experiments, mice were subjected to 45 min left ventricular ischemia followed by 24 h reperfusion. Then prodrugs or vehicles were administered into the left ventricular lumen at 22.5 min of myocardial ischemia. Compared to vehicle-treatment alone, mice treated with prodrugs displayed a significant reduction in circulating levels of cardiac troponin I and myocardial infarct size per area-at-risk, suggesting that perthiol H2S prodrugs indeed exhibit cardiac protection in MI/R injury. It should be noted that the reaction between prodrugs and cysteine yields many reactive sulfane sulfur species. Therefore, further studies on H2S-related and sulfane sulfur-related mechanisms are still needed.

Based on similar strategies, Calderone and coworkers reported dithiohexanoylanhydride as a thiol-activated H2S prodrugs in 2013. The acylpersulfides were proposed to be key intermediates. The H2S-releasing mechanism is the same as that of perthiol H2S prodrugs (Scheme 6).

2.3.2. Photo-induced H2S prodrugs

The second type of controllable H2S prodrugs is light-activated H2S prodrugs. Recently, there have been two example publications. The first one is gem-dithiol-based-H2S prodrugs. In 2013, Xian and coworkers identified geminal-dithiol (gem-dithiol) as a structure, which could release H2S in aqueous solution. Then a photo-cleavable structure (a 2-nitrobenzyl group) was introduced to protect the gem-thiols group. When the molecules were exposed to UV-light, gem-dithiols were regenerated. Subsequent hydrolysis leads to H2S release. Based on this strategy, several gem-dithiol-based H2S prodrugs were prepared. Methylene Blue assay indicated that 200 μmol/L prodrugs could generate a peak concentration of 36 μmol/L H2S under UV irradiation (365 nm). However, there are two

![Scheme 4](image-url)
obvious drawbacks of these prodrugs. First, H₂S release rate depends on the hydrolysis of gem-dithiol, which is nearly fixed. Second, the reactive byproducts 2-nitrosobenzaldehyde can react with H₂S, which results in diminishing H₂S generation. Later, Nakagawa’s group investigated another type of photo-induced H₂S prodrugs: ketoprofenate-caged H₂S prodrugs (Scheme 7). Upon UV irradiation (300–350 nm) for 10 min, 500 μmol/L of the prodrug would generate 30 μmol/L of H₂Si n fetal bovine serum together with 2-propenylbenzophenone and CO₂. These two photo-induced H₂S prodrugs successfully demonstrated the photo-triggering concept, but the cytotoxicity induced by UV-light could limit their applications.

2.3.3. Thiolamino acid
Thiolamino acids as the third class of controllable H₂S-releasing prodrugs were first reported by Giannis and coworkers in 2012. Thioglycine and thiovaline were shown to release H₂S in the presence of bicarbonate under physiological conditions. The mechanism is shown in Scheme 8.

The thiolamino acids interacted with bicarbonate to form carbamate intermediates, which undergoes a cyclization reaction leading to N-carboxyanhydride and H₂S release (Scheme 8). ¹H NMR spectroscopy studies were carried out to measure the decomposition of thioglycine in the presence of NaHCO₃. In a 40 mmol/L bicarbonate solution at 40 °C, 35% N-carboxyanhydride were formed in 72 h. Since there is a high bicarbonate concentration (27 mmol/L) in blood at physiological pH, thiolamino acids can be an H₂S prodrug candidate. Giannis and coworkers compared the H₂S-releasing capacities of thiolamino acids with that of GYY4137. About 50 μmol/L H₂S from 100 μmol/L of thioglycine could be detected by a fluorescent probe dibromobimane, while GYY4137 liberated less H₂S at the same condition. Giannis and coworkers also tested the pharmacological benefits of such H₂S prodrugs. Results showed that thioglycine and thiovaline could enhance intracellular cyclic guanosine monophosphate (cGMP) concentration and promote vasorelaxation. One possible limit of the bicarbonate activated H₂S prodrugs stems from the reactivity of thiolamino acids, which could quickly undergo amidation reaction under aerobic conditions (Scheme 9).
3. Conclusions
The review gives a brief summary of the current state of H₂S prodrugs. These prodrugs not only play an important role as research tools but also are promising candidates for the development of therapeutic agents. All prodrugs have their advantages, and also limitations. The most challenging in this field is still the development of prodrugs with precise control of the release kinetics so that they mimic endogenous H₂S generation. The effects of prodrugs themselves and the byproducts need to be taken into consideration in all the biological experiments. Thus, new hydrogen sulfide prodrugs with improved control of release kinetics are needed in this field.

References

375

Hydrogen sulfide prodrugs


