COMPREHENSIVE INVITED REVIEW

A Review of Hydrogen Sulfide Synthesis, Metabolism, and Measurement: Is Modulation of Hydrogen Sulfide a Novel Therapeutic for Cancer?

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Abstract

Significance: Hydrogen sulfide (H₂S) has been recognized as the third gaseous transmitter alongside nitric oxide and carbon monoxide. In the past decade, numerous studies have demonstrated an active role of H₂S in the context of cancer biology.

Recent Advances: The three H₂S-producing enzymes, namely cystathionine γ -lyase (CSE), cystathionine β -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3MST), have been found to be highly expressed in numerous types of cancer. Moreover, inhibition of CBS has shown anti-tumor activity, particularly in colon cancer, ovarian cancer, and breast cancer, whereas the consequence of CSE or 3MST inhibition remains largely unexplored in cancer cells. Intriguingly, H₂S donation at high amounts or a long time duration has also been observed to induce cancer cell apoptosis *in vitro* and *in vivo* while sparing noncancerous fibroblast cells. Therefore, a bell-shaped model has been proposed to explain the role of H₂S in cancer development. Specifically, endogenous H₂S or a relatively low level of exogenous H₂S may exhibit a pro-cancer effect, whereas exposure to H₂S at a higher amount or for a long period may lead to cancer cell death. This indicates that inhibition of H₂S biosynthesis and H₂S supplementation serve as two distinct ways for cancer treatment. This paradoxical role of H₂S has stimulated the enthusiasm for the development of novel CBS inhibitors, H₂S donors, and H₂S-releasing hybrids.

Critical Issues: A clear relationship between H₂S level and cancer progression remains lacking. The possibility that the altered levels of these byproducts have influenced the cell viability of cancer cells has not been excluded in previous studies when modulating H₂S producing enzymes.

Future Directions: The consequence of CSE or 3MST inhibition in cancer cells need to be examined in the future. Better portrayal of the crosstalk among these gaseous transmitters may not only lead to an in-depth understanding of cancer progression but also shed light on novel strategies for cancer therapy. *Antioxid. Redox Signal.* 31, 1–38.

Keywords: hydrogen sulfide, cancer biology, cancer treatment, CBS inhibitors, H₂S donors, H₂S-releasing hybrids

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Table of Contents

I. Introduction	3
II. Biochemistry of H ₂ S	3
A. Physical and chemical properties of H ₂ S	3
B. Endogenous production of H ₂ S in mammals	4
1. Enzymatic production of H ₂ S	5
a. Cystathionine- β -synthase	5
b. Cystathionine- γ -lyase	5
c. 3-Mercaptopyruvate sulfurtransferase	5
2. Non-enzymatic production of H ₂ S	6
C. H ₂ S catabolism	6
1. Oxidation	6
2. Methylation	6
3. Expiration	6
D. Concentration and quantification methods of H ₂ S in mammals	7
1. Concentration of H ₂ S in mammals	7
2. Quantification methods of H ₂ S in mammals	7
a. Methylene blue method	7
b. MBB method	7
c. S ²⁻ sensitive electrodes method	7
d. Polarographic H ₂ S sensor method	7
e. GC method	8
f. Fluorimetric probes	8
III. H ₂ S-Producing Enzymes in Cancer	8
A. CBS in cancer	8
B. CSE in cancer	9
C. 3MST in cancer	10
IV. Dual Role of H ₂ S in Cancer Biology	10
A. Cancer-promoting effect of H ₂ S	10
1. H ₂ S is a bioenergetic stimulator	10
2. H ₂ S is a pro-angiogenic mediator	11
a. H ₂ S-mediated persulfidation of K _{ATP} channel	12
b. H ₂ S in hypoxia induces angiogenesis	12
3. H ₂ S activates anti-apoptotic pathways	12
a. H ₂ S-mediated persulfidation of NF- κ B p65	13
b. H ₂ S-mediated persulfidation of Keap1	13
c. H ₂ S-mediated persulfidation of MEK1	13
4. H ₂ S accelerates cell cycle	14
B. Anti-cancer effect of H ₂ S	14
1. H ₂ S donors induce uncontrolled cellular acidification	14
2. H ₂ S suppresses cell survival signaling pathways	15
3. H ₂ S induces cell cycle arrest	15
C. Bell-shaped model for the dual role of H ₂ S in cancer	15
V. Crosstalk of H ₂ S with Other Gaso-Transmitters in Cancer Biology	16
A. Crosstalk of H ₂ S and NO in cancer	16
1. Endogenous H ₂ S and NO serve as mutual functional effector molecules	16
2. Bioactive products of H ₂ S and NO interaction possess anti-cancer effect	17
B. Crosstalk of H ₂ S with CO in cancer	17
1. CO-CBS-H ₂ S axis in cancer regulation	17
VI. The Potential of Targeting H ₂ S for Cancer Treatment	18
A. Treatment of cancer with H ₂ S biosynthesis inhibitors	18
1. CBS inhibitor aminooxyacetic acid shows great potential in cancer therapy	18
2. Advances in the development of selective CBS inhibitors	19
B. Treatment of cancer with H ₂ S donors	20
1. Sulfide salts	20
2. Phosphorodithioate derivatives	20
3. <i>Allium sativum</i> extracts and derivatives	21
4. H ₂ S-releasing hybrids	21
a. H ₂ S-releasing non-steroid anti-inflammatory drugs	21
(1) H ₂ S-releasing non-steroid anti-inflammatory drugs possess anti-cancer activity	21

(2) Mechanisms of action of H ₂ S-NSAIDs in cancer inhibition	21
b. NOSH compounds as anti-cancer agents	23
C. The therapeutic potential of H ₂ S donation for cisplatin nephrotoxicity	23
VII. The Novelty and Challenges of H ₂ S-Based Cancer Therapy	24
A. The novelty of H ₂ S-based cancer therapy	24
B. The challenges of H ₂ S-based cancer therapy	24
VIII. Future Directions	25
A. Relationship between H ₂ S level and cancer progression	25
B. Test of drug-like H ₂ S donors in cancer	25
C. Understand the molecular mechanisms underlying H ₂ S effects	25
D. Confirm H ₂ S-linked persulfidation of target proteins	25
E. Crosstalk of H ₂ S with NO in cancer	25
F. Inorganic polysulfide accounts for the anti-cancer effect of H ₂ S?	26
G. A new regulatory circuit of thioredoxin and H ₂ S by controlling persulfidation in cancer?	26
H. H ₂ S-mediated immune cell regulation in cancer progression and therapy	26
IX. Concluding Remarks	26

I. Introduction

HYDROGEN SULFIDE (H₂S) is a colorless gas characterized with a strong rotten egg smell under standard conditions of temperature and pressure. It has been more than 300 years since the first description of H₂S as a poisonous molecule (18). For instance, it has been recorded that heavy exposure to H₂S (>500 ppm) causes unconsciousness and death in humans (238). Usually, the intoxication of H₂S is ascribed to its strong suppressive effect on several critical enzymes in humans such as cytochrome *c* oxidase (238), Na⁺/K⁺ ATPase (238), carbonic anhydrase (205), and monoamine oxidase (299).

Nonetheless, the physiological importance of H₂S is suggested by the fact that mammalian cells are able to actively produce this gaseous molecule (71, 240, 264). This was first demonstrated by Abe and Kimura in 1996 (1) showing that H₂S is an endogenous modulator in the central nervous system. Subsequently, H₂S has been revealed to participate in the regulation of various physiological and pathological conditions within mammalian systems, including central nervous (1), cardiovascular (89), renal (284), reproductive (293), respiratory (83), and digestive systems (64). It is now well recognized as a third endogenous gaso-transmitter along with nitric oxide (NO) and carbon monoxide (CO).

Intriguingly, very recent evidence has accumulated to show that H₂S has a previously unrecognized role in cancer biology. In this review, the roles of H₂S in cancer development and the underlying mechanisms will be surveyed. Moreover, our review will also discuss the progress and the therapeutic potential of H₂S-based molecules for cancer therapy.

II. Biochemistry of H₂S

A. Physical and chemical properties of H₂S

Under ambient temperature and pressure, H₂S is a colorless and flammable gas with a strong rotten egg smell. Acute exposure to high amounts of H₂S (>500 ppm) can lead to human death (238). H₂S is readily dissolved in water with a solubility of about 80 mM at 37°C (121). In aqueous solutions, H₂S is a weak acid and quickly reaches the equilibrium

of H₂S/HS⁻/S²⁻ species. It is estimated that 14% of the sulfide will be H₂S and nearly 86% will be HS⁻ in plasma where the pH value is around 7.4 (311). Nevertheless, changes of pH value can readily alter the equilibrium of H₂S/HS⁻/S²⁻ species. Unless elsewhere specified, in this review, we use the term H₂S to reflect the sum of the species H₂S/HS⁻/S²⁻ and intermediates formed from these, present at physiological pH (306).

Due to the volatile nature of H₂S, it can be easily predicted that the equilibrium will constantly shift to the left, which results in the formation of H₂S evading from the solution (H₂S ↔ H⁺ + HS⁻ ↔ H⁺ + S²⁻). In fact, there are published reports showing that half of H₂S can escape from medium in 5 min in tissue culture wells and in an even shorter time in a bubbled tissue bath (62, 101, 208). This may have partially contributed to the miraculous variations on the reported concentration of H₂S in tissues, plasma, and certain experiments (84, 249, 311). In addition, it is notable to point out that H₂S, as a low-molecular-weight lipophilic gaseous molecule, is freely diffusible across cell membranes without the need of a specific transporter (109, 176).

Sulfur is a multifaceted atom with multiple oxidation states such as -2 in H₂S, 0 in S₈, +2 in SO, +4 in SO₃²⁻ and +6 in SO₄²⁻. As clearly seen from the earlier described fact that -2 in H₂S is the lowest oxidation state of sulfur, therefore H₂S is a reductant and can only be oxidized. As a result, H₂S (and/or intermediates derived from it) can readily modify biological pathways and elicit biological effects *via* the following reactions. The reactions between H₂S and the reactive oxygen species (ROS) or reactive nitrogen species (RNS) species have been extensively studied in solutions (306–308). Nevertheless, Olson *et al.* (211) recently showed that the reaction of H₂S with H₂O₂ is way too slow to elicit any biological significance in mammalian systems. Therefore, it is unlikely that H₂S serves as a direct ROS scavenger in physiological conditions (43). Besides ROS/RNS species, another primary target of H₂S in mammalian systems would be biological irons, namely the metal centers on proteins (199).

In recent years, numerous studies revealed a process called persulfidation as the third H₂S-mediated interaction by which H₂S can react with protein thiol groups and forms

protein persulfides that may induce functional changes of target proteins (194). However, one should bear in mind here that H_2S reacts with R-SOH rather than free thiols since conversion of R-SH to R-SSH is associated with oxidation rather than chemical reduction of sulfur. Similar with other modifications of protein thiols such as *S*-nitrosation, *S*-sulfenylation, and *S*-glutathionylation, H_2S -mediated persulfidation has been found in several proteins and demonstrated to account for the plethora of H_2S -mediated signaling transduction (195, 225). Nevertheless, it should be noted that several critical issues are still awaiting resolution in the field (41, 78). For instance, (i) reliable and proper quantification methods are to be developed; (ii) the mechanisms underlying such modification needs to be thoroughly studied; and (iii) it is required to understand the actual impact of persulfidation on cellular functions.

Interestingly, a fourth H_2S -mediated interaction was recently reported to be the chemical reduction of protein disulfide bonds (220, 289), whereas physiological relevance of

the reaction still awaits further demonstration. For a thorough description of the chemical basis of the interactions mentioned earlier, it is advised to refer a recent review from Filipovic *et al.* on the chemical foundation of H_2S biology (79).

B. Endogenous production of H_2S in mammals

The first description of H_2S production in life can be retrieved back to 1895 in bacteria (215). One hundred years later, the detection of sulfide was first reported in brain tissues (93) and later in other mammalian systems (107, 124). Mammalian cells rely on both enzymatic and non-enzymatic pathways to generate H_2S (Fig. 1).

By using an enzymatic system, H_2S production can be regulated tightly and precisely in mammalian cells, in which H_2S may serve as an endogenous signaling molecule. Among the enzymes, cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) are pyridoxal 5'-phosphate (PLP)-dependent enzymes and are located in the cytosol, whereas PLP-independent

FIG. 1. Endogenous H_2S production in mammalian systems. Mammalian cells mainly rely on the enzymatic pathways to generate H_2S ; however, nonenzymatic pathways are also responsible for a small portion of the production. There are four enzymatic pathways for the biosynthesis of H_2S , including CBS, CSE, 3MST coupled with CAT, and 3MST coupled with DAO. The first three pathways utilize L-cysteine as a main precursor of H_2S , whereas peroxisome-located DAO (blue circle) can catalyze D-cysteine into 3MP, which can be further transformed into H_2S by 3MST. This D-cysteine pathway may exclusively exist in brain and kidney. In addition, CBS and CSE may generate H_2S in the cytosol whereas 3MST mainly resides and synthesizes H_2S in mitochondria (purple rectangle). A small portion of endogenous H_2S is derived via nonenzymatic reduction (dotted green rectangle). In the presence of reducing equivalents such as NADPH and NADH, reactive sulfur species in persulfides, thiosulfate, and polysulfides are reduced into H_2S and other metabolites. 3MP, 3-mercaptopyruvate; 3MST, 3-mercaptopyruvate sulfurtransferase; CAT, cysteine aminotransferase; CBS, cystathionine β -synthase; CSE, cystathionine γ -lyase; DAO, D-amino acid oxidase; H_2S , hydrogen sulfide; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate. Color images are available online.

3-mercaptopyruvate sulfurtransferase (3MST) mainly resides and generates H₂S within mitochondria. Nevertheless, CBS and CSE can translocate into mitochondria when the activity of 3MST is significantly suppressed in oxidative conditions such as hypoxia (82, 276). CBS and CSE also play a role in trans-sulfuration and reverse trans-sulfuration pathways that are responsible for homocysteine metabolism.

Moreover, it is worth pointing out that the mechanisms underlying the regulation of these enzymes such as the expression and activities remain largely unsettled regardless of physiological or pathophysiological conditions. Interestingly, Olson *et al.* (212) recently showed that H₂S may be generated from thioredoxin by catalase using nicotinamide adenine dinucleotide phosphate (NADPH) in hypoxia *in vitro* but this has yet to be demonstrated *in vivo* or in mammalian cells. This could be very relevant to solid tumors where O₂ is low and NADPH is elevated (23).

1. Enzymatic production of H₂S

a. Cystathionine-β-synthase. CBS (EC 4.2.1.22) was first identified in 1969 by Braunstein *et al.* (28) and was later found to be able to produce H₂S mainly through catalyzing L-homocysteine and L-cysteine to form L-cystathionine and H₂S, a reaction analogous to that catalyzed by this enzyme in the trans-sulfuration pathway (29). In the presence of L-cysteine, CBS generates H₂S via a β-replacement reaction along with the production of L-serine. When L-homocysteine is available, the rate of H₂S generation can be accelerated by 23-fold compared with that from L-cysteine alone (257). In addition, CBS is also well known to catalyze the condensation of homocysteine and L-serine, which results in the formation of L-cystathionine and H₂O (118). This reaction is a critical step for the biosynthesis of L-cysteine, which can be further used as an H₂S-producing substrate.

The activity of CBS is found to be mainly regulated by three sites on the protein, including a heme group at the N-terminus, an S-adenosyl-L-methionine (SAM)-binding domain at the C-terminus, and an S-glutathionylation site at cysteine 346. For instance, the modification of CBS carrying the Fe (II) form of heme by CO inhibits CBS activity and H₂S generation (274) and is discussed in section V, Crosstalk of H₂S with Other Gaso-Transmitters in Cancer Biology. In contrast, both SAM and S-glutathionylation lead to the activation of CBS and enhanced production of H₂S (206, 247). In addition, Agrawal and Banerjee have shown that purified CBS protein can be targeted by SUMO *in vitro* and the resulting SUMOylation of CBS leads to the reduction of its activity (2). However, the presence and possible functional consequences of this SUMOylation are still not clear within cells and mammalian systems. The combination of these modulations may allow the quick adjustment of the endogenous H₂S level to cope with various cellular conditions. However, the molecular mechanisms underlying these modulations remain to be addressed in future.

CBS is regarded as the predominant H₂S synthase in the central nervous system, although its expression has also been observed in various types of other organs such as kidney, liver, lymphocytes, uterus, placenta, and pancreas islets (1, 125, 224). Recently, this notion seems to be challenged by a report that brain homogenates of CBS-knockout mice produce a similar level of H₂S to that obtained from wide-type

mice (253). However, we propose that the conflict may result from compensatory expression of 3MST in the brain when CBS is knocked out, which may help maintain the endogenous level of H₂S for neuronal cell survival. As a result, CBS knockout mice-derived brain lysates produce a comparable level of H₂S with that obtained from wide-type mice. Therefore, the data perhaps do not necessarily contradict with the existing notion that CBS is predominant for H₂S production in the brain, particularly in physiological conditions. In this case, more experiments are needed to test the hypothesis whether such possible compensatory mechanisms among H₂S-producing enzymes cause the contradiction.

b. Cystathionine-γ-lyase. CSE (EC 4.4.1.1), similar to CBS, utilizes homocysteine as a substrate to generate H₂S along with α-ketobutyrate and ammonia. Alternatively, CSE can also catalyze L-cysteine to produce H₂S and other by-products such as pyruvate, and ammonia. Using the dithio-bisnitrobenzene assay, Chiku *et al.* (53) have found that at a physiological concentration of L-cysteine and homocysteine, around 70% of H₂S is produced from CSE-mediated α- and β-elimination of L-cysteine whereas CSE-catalyzed α, γ-elimination of homocysteine contributes merely about 29% to the total H₂S content. However, about 90% of H₂S is derived from α, γ-elimination of homocysteine when homocysteine level is increased to the amount comparable with that of hyperhomocysteinemia (53). These suggest that the relative concentration of homocysteine and L-cysteine may determine the main substrate used by CSE for the production of H₂S in mammalian cells.

The activity of CSE is known to be influenced by intracellular Ca²⁺ concentration (182, 343). Specifically, low levels of intracellular Ca²⁺ constitutively induce the production of H₂S by CSE whereas CSE activity is suppressed on the rise of intracellular Ca²⁺ by cell stimulation even in the presence of PLP. Further study has revealed that the regulation is independent of calmodulin as neither calmodulin nor its selective inhibitor W-7 alters the level of H₂S derived from purified CSE from the liver (182). Nevertheless, the precise mechanism underlying Ca²⁺-mediated regulation of CSE activity remains to be defined in future.

In comparison to CBS, CSE distributes more widely in mammalian tissues. It is abundantly expressed in the cardiovascular system and respiratory system (107, 344). CSE may also be the dominant H₂S-producing enzyme in tissues such as liver, kidney, uterus, and pancreatic islets (135, 327, 344). A detectable level of CSE mRNA has been reported in the brain (71, 89); however, it seems that the presence of CSE may not be related to H₂S production in the organ because CSE inhibitors do not alter the production of H₂S in the brain (1).

c. 3-Mercaptopyruvate sulfurtransferase. 3MST is the most recently discovered H₂S-producing enzyme (253). With this enzyme system (Fig. 1), L-cysteine has to be first converted into 3-mercaptopyruvate (3MP) by cysteine aminotransferase (CAT). Thereafter, 3MST transfers a sulfur atom from 3MP onto itself, which leads to the formation of persulfide. H₂S is then released from the persulfide in the presence of a reductant such as thioredoxin (197, 248, 323). Recently, another source of 3MP was found in mammals by Shibuya *et al.*, D-cysteine (251). Specifically, D-cysteine is

transformed into 3MP by peroxisome-located D-amino acid oxidase (DAO). Metabolite exchanges between peroxisome and mitochondria can import 3-MP into mitochondria where it is further catalyzed into H₂S by 3MST. Because of the exclusive location of DAO in the brain and kidney, this H₂S-generating pathway is currently believed to uniquely exist in the two organs.

Distinct from CBS and CSE, the activity of 3MST appears to be regulated intrinsically by its redox state rather than interactions with other factors. Based on its crystal structure, three redox-sensitive cysteines (Cys154, Cys247, Cys263) have been identified on its catalytic site (196). In line with this, oxidative stress significantly suppresses the activity of 3MST and, therefore, H₂S production probably by oxidation of these protein thiols (36, 133). Moreover, modulation of CAT or DAO activity can also apparently affect the generation of H₂S by 3MST. For instance, extensive evidence has shown that aspartate, an inhibitor of CAT, largely inhibits H₂S biosynthesis and is commonly used as a pharmacological tool to study the function of 3MST-derived H₂S (4, 252, 253) whereas one should bear in mind that aspartate is not a selective 3MST inhibitor. Therefore, special caution should be given when studying 3MST-mediated cellular H₂S production due to the fact that multiple enzymes are participating in the process, particularly in organs such as the brain and kidney. In addition, increased intracellular Ca²⁺ has been shown to suppress the activity of CAT and the subsequent H₂S synthesis independently of calmodulin (134, 181). However, the precise molecular mechanism underlying this regulation is unknown.

3MST, similar to CBS and CSE, is expressed in many tissues with particularly high activity in cardiac cells, pericentral hepatocytes in the liver, and the proximal tubular epithelium of the kidney (252, 253, 312). Recent evidence shows that 3MST may be crucial for H₂S generation in the brain as brain homogenates of CBS-knockout mice produce a similar level of H₂S to that of wide-type mice (253). Within mammalian cells, 3MST is mainly located in mitochondria, although a detectable level of 3MST has also been reported in the cytoplasm (134). Nevertheless, it is believed that the occurrence of H₂S generation by 3MST is mainly in mitochondria, because the concentration of L-cysteine is three times higher in the mitochondria than that in the cytoplasm (197).

2. Non-enzymatic production of H₂S. A portion of endogenous H₂S is derived from sulfane sulfur *via* non-enzymatic chemical reduction. In general, the process requires reducing equivalents such as NADPH and nicotinamide adenine dinucleotide (NADH), which are supplied by oxidation of glucose *via* glycolysis or from phosphogluconate *via* NADPH oxidase (244). In the presence of such equivalents, reactive sulfur species in persulfides, thiosulfate, and polysulfides can be reduced into H₂S and other metabolites (210). Essentially, all the components of this non-enzymatic route are available in mammals including reducible sulfur, suggesting the necessity of this pathway in mammalian systems. In accordance with this, hyperglycemia is demonstrated to promote H₂S generation by enhancing this pathway (296).

C. H₂S catabolism

Along with the production of endogenous H₂S, it has to be disposed very quickly as the accumulation of H₂S may result

in organ toxicity. In comparison to the biosynthesis of H₂S, the catabolism of this gaseous transmitter is not well studied and most evidence was obtained by using exogenous H₂S. Therefore, further validation is needed in experimental settings of physiological conditions. Herein, we will discuss three catabolic routes of H₂S in mammalian systems, namely oxidation, methylation, and expiration. It is worth mentioning that hemoglobin also interacts with H₂S, resulting in the formation of bound sulfur in plasma as sulfhemoglobin (45); however, this may serve as an internal storage of sulfide rather than its catabolism and is, therefore, not included here in this review.

1. Oxidation. The vast majority of H₂S is disposed *via* the kidney in the form of sulfate. In the process of sulfate formation, oxidized H₂S combines with another molecule of H₂S and forms one molecule of thiosulfate in mitochondria. This step does not require any enzyme and has been demonstrated within isolated rat kidneys and livers (13, 165). H₂S can also be oxidized by sulfide quinone oxidoreductase (SQOR) in mitochondria to generate persulfide, which is then catalyzed into thiosulfate by rhodanese (115, 116). Recently, Bostelar *et al.* (26) discovered that ferric hemoglobin is also able to oxidize sulfide to thiosulfate, whereas the physiological significance of this reaction still awaits further exploration. Subsequently, conversion of thiosulfate into sulfate and/or sulfite occurs in the presence of sulfide-detoxifying enzymes such as rhodanese (228). The produced sulfite is also quickly oxidized into sulfate (152). To this end, sulfate can be excreted *via* urine as a main product of H₂S catabolism.

However, it should be noted that the urinary concentration of sulfate does not serve as an accurate marker for endogenous H₂S level; sulfate in urine can also be derived from the direct oxidation of L-cysteine (143, 158). Although the oxidation of H₂S may occur in all types of mammalian cells, the primary site of this oxidation is postulated to be in the liver (85).

2. Methylation. Unlike H₂S oxidation, the methylation of H₂S mainly occurs in the cytosol rather than mitochondria. In the process, H₂S is methylated into methanethiol, which can be further methylated into non-toxic dimethylsulfide by thiol S-methyltransferase (305). Compared with sulfide oxidation, the methylation of sulfide is shown to be ~10,000 times slower in colonic mucosa (152). Therefore, it may dispose minimal amounts of H₂S in physiological conditions.

3. Expiration. H₂S can also escape from mammalian lungs. The amounts of exhaled H₂S can be readily detected along with administration of sodium sulfide (Na₂S) (113) or inhibition of endogenous NO synthesis (229). Therefore, it seems that expiration may provide a disposal route of H₂S when large amounts of H₂S are generated. In support of this notion, the exhalation of H₂S is detectable in pathological conditions such as septic shock, hemorrhagic shock, and chronic obstructive pulmonary disease when H₂S is generated excessively (110, 189, 263). However, it remains unknown how much H₂S is lost through lungs in healthy conditions since there is not enough to pick up for measurement. Considering that the free level of H₂S is extremely low in mammalian blood (84, 209), the loss of H₂S *via* this route should be very minimal if there is any.

D. Concentration and quantification methods of H₂S in mammals

1. Concentration of H₂S in mammals. In spite of definitive evidence showing the presence of H₂S in mammalian systems, the exact concentration of H₂S *in vivo* has been in debate for a long time due to conflicting data reported. Using the methylene blue method, initial literatures reported that the concentration of H₂S is above 35 μ M and 50–160 μ M in mammalian plasma and brain, respectively (93, 242, 344, 349). However, later studies have shown that the high concentration of H₂S may be caused by the usage of a strong acid in the measurement (114, 311), because H₂S is released from acid-labile sulfur in the presence of a strong acid when using the methylene blue method.

With the exclusion of a strong acid, 0.7–3 μ M sulfide has been reported in the mammalian plasma utilizing monobromobimane (MBB) method with or without dithiothreitol (111, 249, 282, 315) by more robust analytical techniques such as high-performance liquid chromatography (HPLC). Nevertheless, one should bear in mind that MBB reacts and removes HS[−] from solution, which may result in the release of more HS[−] from some proteins ionized by HS[−] at cationic sites (99).

In contrast, the polarographic sensor method can be used to measure H₂S concentration without sequestering it by which a low nano-molar range of H₂S concentration has been observed in rodent plasma (311, 315). In line with this, it has recently been shown that the concentration of H₂S is about 15 nM in mouse plasma by a newly developed gas chromatography (GC) method (151). Taken together, the concentration of sulfide is suggested to be in the range of nanomolar to submicromolar in mammalian plasma in spite of controversy.

Although the concentration of H₂S in mammalian plasma is demonstrated to be rather low, it may be substantially increased in certain microenvironments. This may be due to three possible mechanisms, including: (i) H₂S release from bound sulfur. For example, it has been demonstrated that activation of neurons causes the release of sulfide from the bound sulfur of nearby astrocytes, which stimulates the activity of Na⁺/HCO₃[−] cotransporter and alkalinizes the astrocytes (114). It is likely that other tissues or organs embrace a similar mechanism as the brain considering the spread of bound sulfur across the mammalian body. (ii) Upregulation of H₂S biosynthesis. It has been shown that tissue H₂S biosynthesis is enhanced in conditions such as streptozotocin-induced diabetes (334). In such conditions, it is expected that H₂S will be temporarily concentrated in the microenvironment, particularly around H₂S-producing enzymes before its diffusion or oxidation. (iii) Suppression of H₂S catabolism. During hypoxia, O₂ reduction leads to suppression of H₂S oxidation and subsequent accumulation of H₂S, which has been demonstrated in various tissues/systems such as blood vessels, carotid body, kidney *etc.* (21, 25, 36).

2. Quantification methods of H₂S in mammals. As reviewed earlier, the concentration of H₂S does not always remain constant and it may fluctuate in particular conditions. Therefore, the accurate measurement of H₂S level is crucial for a precise portrayal of the role of H₂S in individual circumstances. Herein, the commonly used quantification methods of H₂S will be briefly overviewed with emphasis on their sensitivity and reliability for measuring the level of

H₂S in biological samples. The readers are also advised to refer to the review by Kevil and coworkers (140) and another review article from Nagy *et al.* (200) discussing in detail the pitfalls that are associated with sulfide quantification.

a. Methylene blue method. In this method, sulfide is first trapped with a metal (usually zinc acetate). The resultant complex reacts with *N,N*-dimethyl-*p*-phenylenediamine and leads to the formation of methylene blue in the presence of Fe³⁺ under acidic conditions. Subsequently, methylene blue is measured at 670 nm with a spectrophotometer. The methylene blue method was initially developed by Fischer (80) for the quantification of H₂S in natural water and thereafter adapted for analyzing non-biological samples such as sewage, food, and gas (6, 117, 231). Ever since the demonstration of H₂S as an endogenous signaling molecule, it has been extensively employed to measure H₂S concentration in blood, tissues, and other biological specimens perhaps due to its easy protocol for usage (1, 77, 209).

However, it is currently believed that this method is not suitable for these biological samples due to the release of H₂S from acid labile sulfide pools under acidic conditions (114, 311), though it may be useful in estimating total sulfide pools. Nevertheless, it may still serve well as an acceptable assay to measure the activity of H₂S-producing enzymes with proper control groups. Furthermore, it is essential to make the standard curves for sulfide calculation under exactly the same conditions (*i.e.*, pH) with the sample solutions (200).

b. MBB method. In the assay, MBB derivates H₂S into sulfide-diamine product in basic conditions, which is subsequently determined with HPLC equipped with a fluorescence detector. The detection limit of this method is 2 nM at optimum conditions, and the good stability of the sulfide-diamine product allows batch storage and analysis (249). Thus, this method is regarded as a highly useful method for sulfide quantification. Nonetheless, examination of publication revealed that the concentration of H₂S measured under pH 9.5 seems to be higher than that under pH 8.0 (249, 315). We speculate that this discrepancy may be caused by the release of H₂S from some bound sulfide under basic conditions. Thus, special caution should be given for pH control when employing this method to compare H₂S levels among different experimental groups. It is worth mentioning that this method can measure the conversion of sulfide into polysulfide, another potential endogenous signaling molecule, as suggested by Nagy and Winterbourn (201).

c. S^{2−}-sensitive electrodes method. In this method, S^{2−}-sensitive electrodes are used to measure the electronic voltage, which can be further calculated as sulfide concentration. It is able to detect H₂S concentration in 1 min with a detection range of 1–100 μ M and is now widely used for the quantification of H₂S in biological samples (328, 344). Since this method does not require sulfide derivation, it, therefore, allows the kinetic measurement of free sulfide concentration (200). Moreover, a polarographic sulfide sensor was developed to overcome the interference of biomolecules and frequent reconditioning (65), which has shown its feasibility to measure free sulfide levels in *in vivo* models (62, 65).

d. Polarographic H₂S sensor method. This method employs polarographic H₂S sensors for the measurement of H₂S

level with Na₂S or sodium hydrosulfide (NaSH) as a standard. The assay is sensitive with a detection limit of nanomolar range under anoxic conditions and is able to provide a real-time measurement of H₂S from biological samples (65, 139). Though the method is well documented as a reliable method for the measurement of H₂S levels by various studies (139, 209), Whitfield *et al.* (311) have recently reported that they cannot detect any sulfide in plasma derived from various animal species. Later, it was suggested that the earlier on successful studies had constructed electrodes with a unique silicone polycarbonate blend membrane that is not commercially available any longer (140). Intriguingly, Faccenda *et al.* (73) recently showed that polydimethylsiloxane membrane is successful in construction of H₂S electrodes and after measurement of H₂S. However, it can be clearly seen that the sensitivity of the method is apparently affected by the materials of H₂S electrodes. In addition, how to preserve the sulfide content for later measurement remains to be settled when using this method.

e. GC method. The method uses an alkylation technique to extract H₂S followed by GC measurement of the H₂S-derived substance. Shigetoshi *et al.* (254) first established the method and successfully detected the sulfide level in rat blood exposed to H₂S. Recently, Furne *et al.* (84) have improved the method *via* combing GC with a chemiluminescence sulfur detector by which the concentration of free H₂S in mouse blood is later reported to be around 15 nM (151). Both studies showed that the detection limit of the improved method is at nanomolar range. Though it may be not as user friendly as aforementioned techniques, this method is evidently sensitive and specific. Nevertheless, it is notable that the method may primarily facilitate quantification of the species of H₂S alone over other species of sulfide, including HS⁻.

f. Fluorimetric probes. In the method, the reducing ability of H₂S serves as the chemical basis for the synthesis of such probes. Compared with the techniques mentioned earlier, fluorescence probes have tremendous advantages on the sensitive and real-time H₂S detection within specific tissues and cellular compartments. Due to this, the development of H₂S-sensitive fluorescence probes is one of the most rapid growing areas in the field of H₂S biology. As a result, numerous such probes have been reported, such as sulfidefluor-1/2 (SF-1/2), dansylazide azide, sulfide-selective fluorescent probe-1/2, and 7-Azido-4-methylcoumarin (96, 166, 227, 236).

However, they, indeed, have some limitations that should be noted when employing these probes: (i) Some of the probes may have limited sensitivity, and, therefore, long time exposure may be needed to catch the changes of H₂S levels; (ii) tissue autofluorescence may disturb the use of some probes that have a similar excitation/emission wavelength (175); (iii) some of the probes may have non-specific interaction with other reducing biological thiols such as cysteine and glutathione (311, 315), which may limit their use, particularly in *in vivo* settings; and (iv) they actually measure the amount of H₂S produced over the duration of exposure as the chemical reactions of H₂S with these fluorophores are essentially irreversible (99).

III. H₂S-Producing Enzymes in Cancer

H₂S is produced in mammalian cells by three major enzymes, including CSE, CBS, and 3MST. Among these en-

zymes, CSE and CBS are located in the cytosol whereas 3MST resides and generates H₂S in mitochondria. Numerous studies have shown altered expression of the enzyme(s) in the progression of various types of cancer cells, which have been reviewed next and summarized in Table 1.

A. CBS in cancer

The pro-cancer effect of CBS has been thoroughly demonstrated in colon cancer. Tu *et al.* first observed the correlation of CpG island methylation in CBS promoter with the occurrence and progression of colon cancer (285), implying a possible role of CBS in the tumorigenesis. After this, the expression level of CBS has been measured in human colon cancer biopsies, colon cancer cell lines, and normal margin mucosa by Szabo *et al.* (266) and Szabo and Hellmich (267). They found that in comparison to normal tissues the expression levels of CBS rather than those of CSE and 3MST are selectively upregulated in colon cancer biopsies and colon cancer cell lines (266, 267). Intriguingly, the proliferation of colon cancer cell line HCT116 was further accelerated on the activation of CBS with SAM (185) or supplementation of NaSH (although <100 μ M) (32). Conversely, genetic silencing or pharmacological inhibition of CBS lessened the proliferation of colon cancer cell lines along with the reduced migration and invasion (266, 267).

These anti-cancer effects of CBS blockage in colon cancer have been further substantiated in several mouse xenograft models (48, 102). Recently, Pagliara *et al.* (219) reported that 5-fluorouracil treatment induces the downregulation of CBS, which may contribute to the following apoptosis in colon cancer cells. Taken together, these results indicate that CBS is a pro-cancer factor in colon cancer development and its inhibition may serve as a promising strategy for colon cancer therapy.

Accumulating evidence also suggests a pro-cancer role of CBS in human ovarian cancer and breast cancer. The expression of CBS is commonly found in primary serous ovarian carcinoma (24) and also ovarian cancer cell lines (47); however, minimal expression of CBS is detected in the non-malignant ovarian surface epithelial cell line OSE (24). siRNA silencing of CBS abrogates tumor growth (24, 47) and increases the sensitivity of ovarian cancer cell lines to chemotherapeutic drug cisplatin (24). The outcome of CBS silencing might be the disruption of mitochondrial ROS production and adenosine triphosphate (ATP) generation, which may result in the inhibitory effect in ovarian cancer cells (24).

In human breast cancer, a more subtle impact of CBS has been suggested. When CBS is silenced with siRNA, it does not alter breast cancer cell proliferation in *in vitro* culture but significantly reduces tumor growth in xenograft models (246). Further study has shown that CBS silencing inhibits breast cancer cell growth in the presence of activated microphages (246), suggesting that CBS-derived H₂S might protect breast cancer cells from the attack of microphage instead of directly promoting cell growth. The pro-cancer effect of CBS in human breast cancer has been further consolidated by a recently reported association between the 844ins68 polymorphism in the CBS gene and the occurrence of breast cancer (87).

In contrast, shRNA knockdown of CBS has been reported to cause rapid growth of human glioma tumor cells both *in vitro* and *in vivo* (270). However, the expression level of other H₂S-

TABLE 1. CHANGES IN THE EXPRESSION OF VARIOUS HYDROGEN SULFIDE-PRODUCING ENZYMES IN VARIOUS TYPES OF CANCER

Cancer type	Cell line/tissues	mRNA or protein level measured	H ₂ S producing enzymes			Ref.
			CSE	CBS	3MST	
Prostate cancer	LNCaP cell	Protein level	Upregulation	Upregulation	Not tested	(95)
	PC3 cell	Protein level	Upregulation	Upregulation	Not tested	(226)
	LNCaP-B cell; human prostate cancer tissues	Protein level	Downregulation	No change	Not tested	(342)
Melanoma	A375, WM35 cells	mRNA and protein level	Upregulation	Not tested	Upregulation	(120)
	A375, Sk-Mel-5, Sk-Mel-28, PES 43 cells	Protein level	Upregulation	No change	No change	(222)
Gastric cancer	SGC-7901 cell	Protein level	Upregulation	Not tested	Not tested	(123)
	Human gastric carcinoma tissues; SGC-7901 cell	mRNA and protein level	Upregulation	Upregulation	Not tested	(338)
Colon cancer	Human cancer specimens; LoVo, HCT116, HT29 cells	Protein level	No change (Human specimens; LoVo) Downregulation (HCT116, HT29)	Upregulation	No change	(266)
Urothelial carcinomas	SW480 cell	Protein level	Upregulation	Not tested	Not tested	(74)
	Human urothelial carcinomas	Protein level	Upregulation	Not tested	Not tested	(86)
Ovarian carcinomas	Human ovarian cancer samples; OV202, SKOV3, A2780 cells	mRNA and protein level	No change	Upregulation	Not tested	(24)
	OV202, A2780, SKOV3, OVCAR3, OVCAR4, OVCAR5 cells	Protein level	No change (OVCAR3; OVCAR5); Downregulation (OV202;A2780; SKOV3;OVCAR4)	Upregulation	No change	(47)
Hepatocellular carcinomas	Hepatocellular carcinomas specimens	mRNA level	Not tested	Downregulation	Not tested	(132)
Breast cancer	HepG2, PLC/PRF/5 cells	Protein level	Upregulation	Not tested	Not tested	(222)
	Human breast cancer samples; Hs578T, MCF7, MDA-MB-468 cells	mRNA and protein level	Not tested	Upregulation	Not tested	(246)
Astrocytoma	U373 cell	Protein level	Not tested	Not tested	Upregulation	(120)
Neuroblastoma	SH-SY5Y cell	Protein level	Not tested	Not tested	Upregulation	(120)
Gliomas	Human brain gliomas sample	Protein level	Not tested	Not tested	Upregulation	(316)

3MST, 3-mercaptopyruvate sulfurtransferase; CBS, cystathionine β -synthase; CSE, cystathionine γ -lyase; H₂S, hydrogen sulfide.

producing enzyme has not been examined in the study. As recent evidence has shown that 3MST is a crucial enzyme for H₂S production in brain cells, particularly when CBS is genetically deleted (253), therefore it is possible that compensatory H₂S generated from 3MST may have promoted the growth of glioma cells on CBS silencing. The expression of CBS is downregulated in human hepatocellular carcinoma and associated with high tumor grade and high Edmondson grade (132); however, further studies are required to clarify the role of CBS in the progression of this type of cancer.

B. CSE in cancer

CSE might be the dominant H₂S-producing enzyme in peripheral tissues as indicated by experiments with mice lacking CSE (328). Increasing evidence suggests that CSE plays a role in the survival of various types of cancers. In prostate cancer, the abundance of CSE is first found in the LNCaP cell (95), which was later confirmed in other cell lines such as PC-3 (226) and LNCaP-B (342). Overexpression of

CSE or H₂S supplementation with NaSH (although 50–200 μ M) apparently induces cancer cell proliferation (226, 342), suggesting a role of the CSE/H₂S pathway in the survival of prostate cancer. A similar effect of CSE has also been observed in melanoma (222) and gastric cancer (123, 338), as either CSE overexpression or H₂S donors leads to cell apoptosis in both of the cancer types. However, whether CSE promotes or inhibits cancer cell survival remains to be determined, since none of the studies examined the consequences of CSE inhibition or deletion in these cell lines.

Nevertheless, CSE has been found to support cell survival in hepatocellular carcinoma (221, 332). Yin *et al.* (332) has shown that the expression of CSE is under the control of the phosphoinositide-3-kinase/protein kinase B (PI3K/AKT) pathway in hepatoma cell lines QGY-7703 and SMMC-7721. CSE knockdown by siRNA significantly inhibits the proliferation of both cell lines, which has been further echoed in HepG2 cells by another group (221). In colon cancer cells, the expression of CSE is under the regulation of the Wnt/ β -catenin pathway (74), which is an essential player in this type

of cancer. Moreover, either siRNA knockdown or pharmacological inhibition of CSE leads to cell death in SW480 and HCT116 (74), suggesting a possible pro-cancer effect of CSE in colon cancer. This has been further supported by the evidence that NaSH (200 μ M) stimulated cancer cell proliferation by activating extracellular signal-regulated kinase (ERK) and AKT pathways in HCT116 and SW480 (32). Recent evidence has also shown that the expression level of CSE is positively associated with urothelial cell carcinoma of the bladder (86). However, the exact role of CSE remains to be investigated in bladder cancer development.

C. 3MST in cancer

3MST is the third enzyme that is responsible for H₂S production in mammals. Distinct from aforementioned cytosol resident CSE and CBS, it locates and produces H₂S in cellular mitochondria (318). Provided mitochondria can use H₂S as an electron donor for ATP production (82, 276), it may be interesting to postulate that 3MST-derived H₂S might be a direct source of energy in certain tumors if the O₂ supply is sufficient. In accordance with this, high levels of 3MST have been discovered in numerous cancer cell lines or tissues, including astrocytoma cell U373 (120), neuroblastoma cell SH-SY5Y (120, 262), melanoma cell A375 and WM35 (120), and gliomas tissue (316). However, no study has shown the consequence of 3MST modulation in cancer. Therefore, the exact role of 3MST in cancer development still remains uninvestigated but will be an interesting area open to explore in the future.

IV. Dual Role of H₂S in Cancer Biology

On the discovery of altered expression of H₂S-producing enzymes, the possible role of H₂S was extensively studied in cancer cells with genetic or chemical approaches to modulate

the level of H₂S. Existing evidence tends to show a dual role of H₂S in cancer progression (summarized below and in Table 2). Nevertheless, before this, one should bear three facts in mind when interpreting the data, including (i) modulation of H₂S-producing enzymes can alter not only the level of H₂S but also other byproducts such as pyruvate, glutathione, and L-homocysteine (169); (ii) some of these inhibitors are not very specific and their effects could result from mechanisms other than inhibiting these enzymes (297); and (iii) use of high micromolar ranges of H₂S concentration is not physiologically relevant (169).

A. Cancer-promoting effect of H₂S

This section provides a summary of cancer-promoting effects of H₂S, including stimulation of bioenergetics, pro-angiogenesis, activation of anti-apoptotic pathways, and acceleration of cell cycle. Moreover, H₂S-linked protein persulfidation that is relevant to cancer biology is also overviewed.

1. H₂S is a bioenergetic stimulator. Historically, H₂S was well known as a suppressor of the mitochondrial respiratory chain due to its inhibitory effect on cytochrome c oxidase (56). However, the fact that some bacteria utilize sulfide for energy production has urged to test whether this scenario also exists in mammals (30). Currently, several layers of evidence suggest that H₂S at low doses serves as a bioenergetic stimulator in mammalian cells. Gubern *et al.* (94) have shown a very high affinity for mammalian mitochondria with sulfide, which allows the use of sulfide as an energetic substrate at low micromolar concentrations. Subsequently, the enzyme consuming H₂S has been identified to be mitochondrial SQOR, which is an independent parallel

TABLE 2. POSSIBLE MOLECULAR MECHANISMS RELEVANT TO THE DUAL ROLE OF HYDROGEN SULFIDE IN CANCER BIOLOGY

Role of H ₂ S	Molecular mechanisms	Possible target proteins/pathways	Interaction mechanisms	Ref.
Cancer promoting	Stimulation of bioenergetics	Sulfide quinone reductase	H ₂ S as a substrate	(145, 186)
		Mitochondrial ATP synthase	Persulfidation	(187)
		Lactate dehydrogenase A	Persulfidation	(286)
		Glucose transporter	Unknown	(163)
	Pro-angiogenesis	K _{ATP} channel activation	Persulfidation	(195, 223)
		PI3K/Akt activation	Unknown	(159)
		MAPKs activation	Persulfidation of MEK1?	(341)
	Anti-apoptosis	NF- κ B pathway activation	Persulfidation of p65	(245)
		Keap1-Nrf2 pathway activation	Persulfidation of Keap1	(329)
		MEK1-ERK activation	Persulfidation of MEK1	(341)
Anti-cancer	Acceleration of cell cycle	ERK activation	Persulfidation of MEK1	(32, 341)
		PI3K/Akt activation	Unknown	(172, 174)
		Glucose transporter	Unknown	(149, 163)
	Induction of uncontrolled cellular acidification	Anion exchanger	Unknown	(149)
		Sodium/proton exchanger	Unknown	(149)
		Suppression of NF- κ B pathway	Unknown	(222)
	Suppression of cell survival	Suppression of STAT3	Unknown	(171)
		Reduction of pleckstrin homology-like domain-A1	Unknown	(193)
	Induction of cell cycle arrest	Upregulation of p21 ^{Cip1}	Unknown	(321)
		Downregulation of cyclin D1	Unknown	(150)

AKT, protein kinase B; ATP, adenosine triphosphate; ERK, extracellular signal-regulated kinase; K_{ATP}, ATP-sensitive potassium; MAPK, mitogen-activated protein kinase; MEK1, ERK activator kinase 1; NF- κ B, nuclear factor kappa-light-chain enhancer of activated B cells; Nrf2, transcription factor NF-E2-related nuclear factor 2; PI3K, phosphoinositide-3-kinase; STAT3, signal transducer and activator of transcription 3.

electron donor to coenzyme Q, in addition to complexes I and II (145, 186).

Endogenous H₂S derived by 3MST plays a pivotal role in supporting the physiological cellular bioenergetic functions, as basal bioenergetic parameters are reduced on siRNA knockdown of 3MST (186, 269). However, they can be enhanced by 3MP (186), the substrate of 3MST. Although stress conditions such as hypoxia were reported to induce the translocation of CSE and CBS into mitochondria to sustain energy production (82), yet the deprivation of O₂ should reduce the production of ATP from H₂S as O₂ serves as the terminal electron acceptor. Furthermore, it has been shown that H₂S metabolism is progressively reduced as PO₂ falls in isolated mitochondria and this occurs at physiologically relevant PO₂ (213). Therefore, H₂S-mediated mitochondrial respiration may be only useful to cancer development when O₂ supply is still sufficient.

Other than serving as a substrate in mitochondrial respiration, H₂S may also stimulate cellular bioenergetics by elevating intra-mitochondrial cAMP levels (188), inducing the persulfidation of mitochondrial ATP synthase (187) and lactate dehydrogenase A (286). Besides mitochondrial respiration, H₂S has also been implicated in the regulation of glycolysis, which is well recognized as the main energy source that cancer cells rely on (287). For instance, an inorganic source of H₂S (using the salt NaSH) enhanced glucose uptake and glycolysis efficiency in cardiomyocytes likely *via* stimulating the activity of glucose transporter (GluT). Subsequently, this finding has been paralleled in a panel of cancer cell lines (163).

In line with these, CBS knockdown significantly diminishes the bioenergetic parameters such as oxygen consumption and ATP production in both colon cancer (102, 266) and ovarian cancer cells (24). Nonetheless, the elaboration of whether and how H₂S enhances the activity of GluT is still required, particularly in cancer cells. Taken together, H₂S stimulates mitochondrial respiration and glycolysis, thereby supporting the energy required by cancer development (Fig. 2).

2. H₂S is a pro-angiogenic mediator. Angiogenesis plays a critical role in cancer development given that it promotes new blood vessel formation, which provides tumors with oxygen and nutrients (44). The pro-angiogenic function of H₂S was initially found in the late 2000s. NaSH, a commonly used experimental source of H₂S, accelerates cell proliferation, migration, and tubule-like structure formation in cultured endothelial cells (31, 223). In addition, exposure of chicken chorioallantoic membrane to NaSH promoted the growth and branching of blood vessels (223). The pro-angiogenic effect of H₂S has further been confirmed in rat models (223). Moreover, either pharmacological inhibition or genetic deletion of CSE blocked vascular endothelial growth factor (VEGF)-induced angiogenesis (223, 233), suggesting that H₂S may act as a physiological angiogenic mediator.

Further studies revealed that the signaling pathways underlying the pro-angiogenic effect of H₂S may involve the PI3K/AKT pathway, the mitogen-activated protein kinase pathway, and ATP-sensitive potassium (K_{ATP}) channels (31, 70, 159, 223). The pro-angiogenic effect of H₂S has recently

FIG. 2. H₂S as a bioenergetic stimulator. H₂S enhances glucose uptake probably by stimulating the activity of GluT and thereby increasing the rate of glycolysis. Meanwhile, H₂S can be consumed by mitochondrial SQR to generate electrons for the mitochondrial respiratory chain. By doing these, H₂S may enhance the production of intracellular ATP and support cancer cell proliferation. ATP, adenosine triphosphate; GluT, glucose transporter; SQR, sulfide quinone reductase. Color images are available online.

FIG. 3. H₂S as a pro-angiogenic mediator. VEGF increases H₂S level by up-regulating the expression of CSE in endothelial cells. Both endogenous and exogenous H₂S (*in vitro*: <200 μM; *in vivo*: <200 μmol/kg/day) serve as stimulators of angiogenesis by activating multiple signaling pathways. For instance, H₂S-mediated persulfidation of the K_{ATP} channel (cysteine-43) leads to its activation and downstream phosphorylation of p38, which causes angiogenesis. In addition, the activation of ERK and AKT is also implicated in the angiogenic effect of H₂S in endothelial cells. AKT, protein kinase B; ERK, extracellular signal-regulated kinase; K_{ATP}, ATP-sensitive potassium; VEGF, vascular endothelial growth factor. Color images are available online.

been observed in endothelial cells of tumors. With a well-established model of tumor angiogenesis, namely endothelial cells obtained from breast carcinomas (B-TECs), Pupo *et al.* (233) found that NaSH (1–10 μM) enhances the migration of B-TECs whereas inhibition of CSE suppresses VEGF-induced migration of B-TECs, suggesting that both exogenous and endogenous H₂S promote angiogenesis in breast cancer. In addition, genetic silencing of CBS diminished the neovessel density and tumor growth in rat models of colon cancer (266) and ovarian cancer (24). These results suggest that H₂S may be beneficial for tumor growth by promoting angiogenesis, thereby delivering nutrients and oxygen to cancer cells (Fig. 3).

Nevertheless, it is still noteworthy that NaSH at high concentrations or doses (*in vitro*: >200 μM; *in vivo*: >200 μmol/kg/day) may also exhibit a suppressive effect on angiogenesis (31, 317), suggesting that the pro-angiogenic activity occurs only with endogenous H₂S and/or exposure of low amounts of exogenous H₂S in the context of cancer biology.

a. H₂S-mediated persulfidation of K_{ATP} channel. K_{ATP} channel has long been recognized as a potential target of H₂S, accounting for its cardiovascular effects such as vasorelaxation, cardioprotection, and hyperpolarization (119, 272, 340, 344). Interestingly, the K_{ATP} channel antagonist glibenclamide attenuates H₂S-induced p38 phosphorylation and migration of endothelial cells (223), suggesting that H₂S–K_{ATP} channel–p38 axis may partially mediate the pro-angiogenic effect of H₂S.

An elegant study from Mustafa *et al.* has recently unclosed that H₂S-mediated persulfidation activates K_{ATP} channel and induces the downstream effects (195). In the study, they have shown that NaSH induces persulfidation of K_{ATP} channels in

K_{ATP} channel overexpressed HEK293 cells. The persulfidation can also be detected in cells overexpressing CSE but not in those lacking CSE, indicating that endogenous H₂S induces the persulfidation of the K_{ATP} channel. Importantly, the physiological relevance of the persulfidation is further demonstrated by the observation that cholinergic stimulation of mouse aorta increases persulfidation of the K_{ATP} channel in wild-type but not CSE-deficient mice. In addition, mutation of cysteine 43 that lies close to the surface of the K_{ATP} channel completely abolished the persulfidation and H₂S-mediated vasorelaxation, indicating that Cysteine 43 is the principal target of persulfidation in K_{ATP} channel.

b. H₂S in hypoxia induces angiogenesis. Hypoxia is recognized as a primary physiological regulator of angiogenesis. Tumor hypoxia occurs when a tumor rapidly grows, resulting in limited supply of oxygen. This leads to the induction of the transcriptional factor hypoxia inducible factor 1α (HIF-1α) and subsequent angiogenesis, which helps bring nutrients and oxygen to the tumor. One is advised to refer to Liao and Johnson's article for a detailed description of the process (164). Notably, hypoxia can profoundly evaluate the level of H₂S because it inhibits the catabolism of H₂S (37) and induces the expression of CSE (294), which is the main H₂S producer in endothelial cells. Interestingly, a series of recent studies also provides strong evidence showing that H₂S promotes ischemia-induced angiogenesis by augmenting the expression of HIF-1α (25, 168, 268). Therefore, it is highly likely that H₂S may partially mediate hypoxia-induced angiogenesis in cancer progression, though direct demonstration of this notion remains lacking.

3. H₂S activates anti-apoptotic pathways. Apoptosis is critical for the maintenance of tissue homeostasis in

multicellular organism; however, it is suppressed in cancer cells, which affords tumor cells the capability of surviving under various stresses. The anti-apoptotic role of H₂S is clearly manifested by several aspects, including (i) mouse embryonic fibroblasts and endothelial cells derived from CSE deficiency mice display accelerated cellular senescence compared with the counterparts from wild-type mice (329, 341); (ii) H₂S supplementation reverses CSE deficiency-induced senescence (329, 341); (iii) inhibition of H₂S biosynthesis induces apoptotic responses in various types of cells (102, 103); and (iv) H₂S shows a protective effect against various apoptotic stimuli (250, 275, 331, 339, 346).

In the context of cancer biology, the anti-apoptotic effect has also been demonstrated in numerous types of cancer cells such as colon cancer (241), hepatomas cancer (345), and neuroblastoma (281). Further studies have revealed the possible underlying mechanisms such as activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) (245), Keap1-transcription factor NF-E2-related nuclear factor 2 (Nrf2) (329), and ERK activator kinase 1 (MEK1)-ERK pathways (341) mediated by H₂S-linked persulfidation (Fig. 4).

a. H₂S-mediated persulfidation of NF- κ B p65. The NF- κ B pathway mediates its anti-apoptotic function by inducing the expression of several anti-apoptotic proteins such as TNFR-associated factor (TRAF)-1, TRAF-2, caspase-8-c-FLP, and cellular inhibitor of apoptosis (207). The activation of the pathway requires the nucleus translocation of p65 (19), whose lacking induces the death of mice at the embryonic stage due to extensive liver apoptosis (20). Tumor necrosis factor (TNF) α , an activator of the NF- κ B pathway, stimulated CSE expression, thereby tripling H₂S generation. CSE gen-

erated H₂S-enhanced DNA binding of p65 and the downstream gene expression, which is absent in mice lacking CSE. As a result, the anti-apoptotic function of the NF- κ B pathway is largely diminished in CSE-deleted mice (245).

Sen *et al.* have recently revealed that H₂S promoted the binding of p65 to the promoters of downstream genes by persulfidation cysteine-38 on p65, as the mutation of cysteine-38 abolishes the effect of H₂S on p65 persulfidation and NF- κ B activation (245). Importantly, the persulfidation is verified by several methods, including biotin switch assay, maleimide assay, and liquid chromatography-mass spectrometry (245). In line with this finding, H₂S has long been suggested to be an endogenous mediator of inflammation by augmenting NF- κ B pathway activity (154, 310, 336). Similarly, inhibition of CSE may diminish cell proliferation of melanoma cells *via* suppression of the NF- κ B pathway (222). Despite this, one should bear in mind that exogenous H₂S may actually be able to suppress the activation of the NF- κ B pathway in inflammatory conditions (36, 38, 83, 92, 155).

b. H₂S-mediated persulfidation of Keap1. The importance of the Keap1-Nrf2 pathway in tumorigenesis can be evidenced by the finding that somatic mutations of Nrf2 or Keap1 are frequently detected in cancer cells with overactivation of Nrf2-mediated transcription (130), which may afford malignant cells the growth advantage by promoting tumor growth and enhancing cancer chemoresistance (146, 344).

Intriguingly, a recent study from Wang *et al.* suggested that H₂S may be an endogenous stimulator of the Keap1-Nrf2 pathway by persulfidation Keap1 at cysteine-151 (329). In the study, they have shown that in comparison with mouse embryonic fibroblasts from wild-type mice the cells obtained from CSE knockout mice display increased oxidative stress and accelerated cellular senescence, which can be reversed by the supplementation of NaSH (329). In addition, they have shown that NaSH can persulfidate Keap1 and the persulfidation can be fully abolished by the mutation of cysteine-151 of Keap1, but not cysteine-288 (329). This indicates that H₂S may persulfidate Keap1 at cysteine 151, which induces the release of Nrf2. Importantly, in CSE wild-type cells, the persulfidation of Keap1 is detectable and can be further enhanced by the H₂S-producing substrate cysteine; however, it is absent in CSE knockout cells (329), suggesting that H₂S may serve as an endogenous activator of the Keap1/Nrf2 pathway.

By activating the Keap1-Nrf2 pathway, H₂S may induce the downstream expression of a battery of cytoprotective genes called vitagens such as glutathione *S*-transferases, epoxide hydrolase, and ferritin, which may afford cancer cells the adaption for chronic oxidative stress during their progression (33–35). Nevertheless, how persulfidation of Keap1 triggers the release of Nrf2 remains unclear and further study is, therefore, needed.

c. H₂S-mediated persulfidation of MEK1. Somatic mutation is one of the most pervasive characteristics of human tumors (106). To overcome the devastating effect of genetic instability, cancer cells activate a batch of responses, including activation of DNA repair pathways (14, 39). Among these pathways, the overactivation of MEK1-ERK-poly [ADP-ribose] polymerase 1 (PARP1) pathway is commonly detected in a broad spectrum of cancer cells whose inhibition serves as a promising strategy for cancer therapy (300).

FIG. 4. H₂S activates anti-apoptotic pathways. H₂S is able to activate multiple anti-apoptotic pathways by H₂S-linked persulfidation. For example, H₂S persulfidates cysteine-38 of the NF- κ B p65 subunit and results in its activation (*middle*); H₂S persulfidates cysteine-151 of Keap1 and induces Nrf2 translocation into the nucleus; and H₂S persulfidates cysteine-341 of MEK1 and induces subsequent phosphorylation of ERK1/2. MEK1, ERK activator kinase 1; NF- κ B, nuclear factor kappa-light-chain enhancer of activated B cells; Nrf2, transcription factor NF-E2-related nuclear factor 2. Color images are available online.

An interesting article from Zhao *et al.* suggested that H₂S may facilitate DNA repair by activating the MEK1–ERK–PARP1 pathway (341). In the study, they found that the activity of PARP1 is significantly lower in CSE knockout kidney, liver, and endothelial cells compared with their wild-type counterparts (341). NaSH treatment apparently activates PARP1 and enhances the capability of DNA damage repair, which can be inhibited by two inhibitors of MEK1 (U0126 and PD98059) that can suppress ERK1/2 phosphorylation (341), suggesting the involvement of MEK1 in H₂S-stimulated ERK1/2 phosphorylation and PARP1 activity. Moreover, the persulfidation of MEK1 is clearly observed in kidney lysates from wild-type mice, which can be further enhanced by the treatment of NaSH (341). The mutation of cysteine-341 not only fully abolishes NaSH-induced persulfidation of MEK1 but also suppresses the phosphorylation of ERK1/2 (341), indicating that H₂S selectively persulfidates MEK1 at cysteine-341 and induces the downstream effects. However, whether cancer cells have higher levels of MEK1 persulfidation is still not clear.

4. H₂S accelerates cell cycle. Cancer cells gain the capability of infinite proliferation through evading cell cycle arrest (100). Recent evidence indicates that H₂S is able to accelerate cell cycle in various types of cells such as endothelial cells, cardiomyocytes, and cancer cells (15, 63, 326). For instance, exogenous application of H₂S (NaSH, 200–500 μ M) reduces the expression of cell cycle regulatory genes such as replication protein A70 and retinoblastoma protein 1 but increases the expression of proliferating cell nuclear antigen and cyclin-dependent kinase 4, thereby promoting cell

proliferation in several oral squamous cell carcinoma cell lines (172).

The acceleration effect of H₂S on cell cycle has also been found in colon cancer cells (32) and hepatoma cells (221). The signaling mechanism underlying the action might be attributed to the activation of the ERK and AKT pathway (32, 172, 174, 221) because inhibition of either ERK or AKT phosphorylation has been revealed to partially abolish the cell cycle accelerating effect of H₂S on squamous cell carcinomas cell lines and colon cancer cell lines (32, 172). Although not directly demonstrated, the underlying mechanisms of H₂S-induced ERK activation may be ascribed to the persulfidation of MEK1 as discussed earlier. However, the molecular mechanism underlying H₂S-induced AKT phosphorylation remains unclear. Considering the crucial role of AKT in human cancer development (216), the deciphering of this will be of great value.

B. Anti-cancer effect of H₂S

In spite of the earlier mentioned evidence that H₂S is beneficial for cancer development, recent studies have also disclosed that H₂S donation especially at high doses or long time duration leads to cancer cell death without affecting the viability of normal fibroblast cells. In this section, the possible mechanisms underlying the anti-cancer effects of H₂S have been reviewed in the context of cancer development (Fig. 5).

1. H₂S donors induce uncontrolled cellular acidification. One hallmark of cancer cells is the utilization of glycolysis as the main pathway for energy production (97). Thus, they tend

FIG. 5. Detrimental effects of H₂S donation against cancer development. (a) H₂S induces uncontrolled cellular acidification. H₂S stimulates the rate of glycolysis by enhancing the uptake of glucose, which leads to the accumulation of lactate and intracellular acid. Due to the inhibitory effect of H₂S on the activity of AE and NHE, the intracellular acid cannot be efficiently exported out of cells, which causes cancer cell death. (b) H₂S suppresses the cell survival signaling pathway. H₂S has been found to suppress the activity of cell survival pathways such as NF- κ B and STAT3 and, therefore, downregulates the downstream anti-apoptotic proteins, which leads to cancer cell death. (c) H₂S induces cell cycle arrest. H₂S induces cell cycle arrest at G1/S by upregulating the expression of p21^{Cip1} and downregulating expression of cyclin D1. Besides, H₂S was also found to induce cell cycle arrest at G2/M; however, the underlying mechanism has not been studied. AE, anion exchanger; NHE, sodium/proton exchanger; STAT3, signal transducer and activator of transcription 3. Color images are available online.

to have enhanced capacity for glucose uptake and its conversion into lactate. Lactate accumulation results in cellular acidification and stress. However, cancer cells can efficiently export intracellular acid out of cells, which helps form an acidic microenvironment promoting angiogenesis and tumor metastasis (90, 243). Therefore, targeting regulators of intracellular pH is recognized to be a promising strategy for cancer treatment (301).

It was found that a slow-release H₂S donor GYY4137 (200–1000 μ M) enhances glycolysis of cancer cells by increasing the uptake of glucose, meanwhile it disrupts the exporting of intracellular acid possibly by suppressing the activity of anion exchanger (AE) and sodium/proton exchanger (NHE) (149) (Fig. 5a). Nevertheless, it should not be neglected that the catabolism of H₂S to H₂SO₄ may also contribute to the subsequent intracellular acidification. As a result, this causes uncontrolled intracellular acidification and subsequent cell death in a panel of cancer cell lines (149). ZYJ1122, a control compound for GYY4137 that cannot release H₂S as it has no sulfur, had no such effect (149, 150), suggesting that the aforementioned activity of GYY4137 may be exclusively derived from H₂S. Intriguingly, GYY4137 exhibits strong anti-tumor effect in mice models (150).

The effect of GYY4137 has also been studied in fibroblast cells such as Wi-38 and MCF10A in their study (149, 150) and GYY4137 did not cause intracellular acidification in these non-cancerous cells. In contrast to this, NaSH (10 μ M–1 mM) was found to decrease the intracellular pH of vascular muscle cells by stimulating the activity of Cl[−]/HCO₃[−] transporter (148). Thereafter, this effect has been reproduced in primary cultured glia cells, yet not in the neuroblastoma cell line SH-SY5Y (170). Therefore, the controversy among these studies needs to be resolved in the future; for instance, whether the discrepancy can be ascribed to (i) the distinct releasing manner of H₂S between NaSH and GYY4137; (ii) the different levels of AE, NHE, and Cl[−]/HCO₃[−] among different types of cells; and/or (iii) non-H₂S effects derived from GYY4137.

2. H₂S suppresses cell survival signaling pathways. Cancer cells disrupt the balance between apoptosis and survival through sustained activation of pro-survival signaling pathways, leading to the constant increase of cancer cell numbers (60). The NF- κ B pathway acts as such a signaling pathway and its abnormal activation has been commonly found in various types of cancers, including non-small lung cancer, breast cancer, prostate cancer, *etc.* (237).

Despite that H₂S may stimulate the NF- κ B pathway as mentioned earlier by persulfidating the p65 subunit, H₂S has been demonstrated to inhibit TNF α and lipopolysaccharides-stimulated NF- κ B activation (92, 309). Therefore, it is not surprising to find that constant exposure of cancer cells to H₂S (222) or its donating hybrids (127) suppresses NF- κ B activity and causes cancer cell apoptosis. However, the molecular target for the inhibitory effect of H₂S on NF- κ B activity has not been identified. In addition, other pro-survival signaling molecules have been reported to account for the anti-cancer effect of H₂S. For instance, GYY4137 causes the apoptosis of hepatocellular carcinoma cell lines by suppression of signal transducer and activator of transcription 3 (STAT3) activation and downregulation of STAT3-mediated downstream proteins such as B cell lymphoma 2, survivin,

and VEGF (171). In addition, constant exposure to H₂S causes oral cancer cell apoptosis, possibly by reducing the expression of pleckstrin homology-like domain-A1, an apoptotic suppressor in this type of cancer (193). In the future, the target proteins of H₂S in cell survival pathways need to be unveiled and studied in detail.

3. H₂S induces cell cycle arrest. Dysregulation of cell cycle has been proved to be involved in cancer progression (72). Thus, induction of cell cycle arrest is effective to treat cancer (173). The suppressive effect of H₂S on cell cycle transition has been reported by numerous studies (Fig. 5c). S-proargyl-cysteine (SPRC), an H₂S donor, causes cell cycle arrest at the G1/S phase in the gastric cancer cell line SGC-7901 and subsequent apoptosis both *in vitro* and *in vivo* (123). NaSH (0.4–1 mM) leads to cell cycle arrest at G1/S, possibly by up-regulating cyclin-dependent kinase inhibitor p21^{Cip1} in a panel of colon cancer cell lines (HT-29, SW116, and HCT116) (321).

The inductive effect of GYY4137 on cell cycle arrest has also been proposed in various cancer types (150, 171). For instance, Lu *et al.* (171) found that GYY4137 suppressed G1/S cell cycle transition by downregulating the expression of cyclin D1 and the tumor growth is inhibited in the subcutaneous HepG2 xenograft model as a result. It was also reported that GYY4137 caused partial G2/M arrest in breast cancer cell line MCF7 whereas the underlying mechanism has not been studied (150). Attractively, H₂S seems to specifically induce cell cycle arrest in cancer cells, as neither NaSH nor GYY4137 caused cell cycle arrest in normal fibroblast cells in the aforementioned studies (150, 321). However, the molecular targets of H₂S eliciting these effects remain unclear.

Due to its pleiotropic effect on biological processes, there are more potential mechanisms likely underlying H₂S-mediated anti-cancer activity. For example, H₂S may increase the level of E-Cadherin possessing anti-metastatic effect (108); and H₂S has also been found to downregulate the level of histone deacetylases that may result in the epigenetic reactivation of tumor suppressor genes (204). These have been summarized elsewhere in an excellent review by Predmore *et al.* (232).

C. Bell-shaped model for the dual role of H₂S in cancer

The role of H₂S in cancer development seems to be controversial. Therefore, an appropriate model system is highly desirable to explain why H₂S exerts diametrically opposite effects in cancer cells, in different laboratories. Recently, Hellmich *et al.* (102) proposed a reasonable bell-shaped model to illustrate the effects of H₂S in cancer cells. In the model (Fig. 6), a bell-shaped curve is employed to describe the effects of H₂S on cancer cell proliferation. In general, endogenous H₂S or a relatively low level of exogenous H₂S exhibits pro-cancer effect. In contrast, exposure to H₂S with high amounts leads to cancer cell death. This is consolidated by the fact that CBS inhibition not only leads to the inhibition of cell growth but also limits the toxic effect of either 3 mM NaSH or 3 mM GYY4137 in the colon cancer line HCT116 (102).

Supportively, Wu *et al.* (317) recently showed that NaSH (25–100 μ M) facilitated vessel formation and tumor growth of hepatocellular carcinomas, both of which were significantly suppressed when the concentration of NaSH was

FIG. 6. The bell-shaped model for the dual role of H₂S in cancer development. A bell-shaped curve is employed to describe the exposure-dependent effect of H₂S on cancer cell proliferation. In general, endogenous H₂S or a relatively low level of exogenous H₂S exhibits pro-cancer effects. In contrast, exposure of H₂S at high amounts or long-time exposure leads to cancer cell death. The models suggest that H₂S biosynthesis inhibition and H₂S donation serve as two distinct ways for cancer treatment. Color images are available online.

increased to 800 μ M. Nonetheless, it is notable that the study relies on a hypothesis that modulating endogenous H₂S production is similar to exogenous H₂S donation, which may still be plausible considering the unknown intracellular and subcellular H₂S concentration after these modulations. Moreover, SAM, a putative allosteric CBS activator, promoted cancer cell proliferation at a lower concentration range (0.1–0.3 mM). However, it inhibited cell proliferation at higher concentrations (1–3 mM) and this effect was partially blocked by CBS silencing (102), suggesting that over-activation of H₂S-producing enzymes can also suppress cancer cell proliferation.

Interestingly, this biphasic dose response of H₂S in cancer cells well reflects a hormetic response that was recently proposed by Calabrese *et al.* (34). Specifically, H₂S at low concentrations may activate endogenous cellular defense mechanisms that lead to cell death at high concentrations due to its toxic effects. In addition, continuous supplementation of NaSH, (5–20 μ M; every 2 h), induced cancer cell death while sparing non-cancerous cells such as MCF10A and Wi-38 after 5 days (150). This effect was well reflected with the slowly releasing H₂S donor GYY4137 (42, 150). These results suggest that long time exposure to H₂S, even at low doses, can also cause cancer cell death.

Taken together, the bell-shaped models may provide a valuable framework to harmonize the controversies regarding the seemingly controversial role of H₂S in cancer development. Meanwhile, it is suggested that H₂S biosynthesis inhibition and H₂S donation serve as two distinct ways for cancer treatment. Nonetheless, further studies are warranted

to precisely define what the exact levels of sulfide exposure are with respect to carcinogenesis in the bell-shaped curve.

V. Crosstalk of H₂S with Other Gaso-Transmitters in Cancer Biology

A. Crosstalk of H₂S and NO in cancer

There is plenty of evidence showing that H₂S and NO actually share some similar actions such as relaxing smooth muscle and interacting with heme groups in protein molecules (271, 290, 296). Intriguingly, these molecules are also demonstrated to crosstalk with each other, even in physiological conditions. Herein, the crosstalk of H₂S with NO is reviewed with an emphasis in the context of cancer biology.

1. Endogenous H₂S and NO serve as mutual functional effector molecules. The similarities in the function of H₂S and NO have urged the investigation whether these two molecules serve as each other's downstream signaling molecule. Up to date, there is adequate evidence to suggest that they may accomplish this by mutually affecting each other's biosynthesis in mammalian cells (Fig. 7). NO is able to induce CSE expression (298, 344) and facilitate the cellular uptake of H₂S-producing substrate L-cysteine (153), thereby enhancing the production of endogenous H₂S.

Conversely, H₂S also enhances the production of NO. For example, CSE deficiency in mice causes endothelial nitric oxide synthase (eNOS) dysfunction, NO reduction, and aggravated myocardial ischemia/reperfusion injury (137). When H₂S is supplemented, NO level increases by the induction of eNOS expression (55, 142, 180). In addition, NO production is also prompted on the persulfidation of eNOS induced by

FIG. 7. Endogenous H₂S and NO serve as mutual functional effector molecules. H₂S and NO mutually enhance each other's biosynthesis in mammalian cells. NO is able to induce the biosynthesis of H₂S by stimulating CSE expression and facilitating the cellular uptake of H₂S-producing substrate cysteine. Conversely, H₂S also enhances the production of NO by stimulating the activity and expression of eNOS. eNOS, endothelial nitric oxide synthase; NO, nitric oxide. Color images are available online.

H₂S (7, 131). As a result, it was found that suppression of eNOS lessens H₂S-mediated vasorelaxation and genetic knockdown of CSE attenuates NO-stimulated angiogenesis (55). In the context of cancer biology, both endogenous H₂S and NO are suggested to be beneficial for cancer progression (265, 314). Therefore, it is likely that NO partially mediates the pro-cancer effect of H₂S and vice versa. This implies that simultaneous inhibition of H₂S and NO biosynthesis may afford synergic effects on the suppression of cancer cell growth, which should be tested in the future.

2. Bioactive products of H₂S and NO interaction possess anti-cancer effect. For a long period, H₂S and NO signaling pathways have been also observed to be intimately intertwined with mutual potentiation of responses, particularly in the cardiovascular system (69, 198). For instance, Yong *et al.* (333) have reported that a combination of H₂S and NO produces positive inotropic and lusitropic effects likely *via* formation of a thiol-sensitive molecule that is suggested to be nitroxyl (HNO) in the study. This is further confirmed by studies published by Filipovic *et al.* (78) and Zhou *et al.* (347). However, the possible interaction between these two gaseous transmitters remains unexplored in the context of cancer biology.

A recent study from Whiteman *et al.* (308) suggests that thionitrous acid (HSNO) is spontaneously formed when H₂S and NO gases are mixed at room temperature in the presence of metallic surfaces. And a further reaction of HSNO with H₂S may lead to the formation of HNO and inorganic polysulfides such as H₂S₂ (202). Subsequently, Cortese-Krott

et al. (59) have identified that the chemical reaction between H₂S and NO occurs even in physiological conditions and the key bioactive reaction products of the interaction are S/N-hybrid species, inorganic polysulfides, and HNO (Fig. 8).

Chemical studies on some of those S/N-hybrid species, however, have strongly questioned the formation of these intermediates in the cells, and more so *in vivo*. For example, Koppenol and Bounds (141) showed that it would take more than 1 day to generate a maximum of 1 nM of SSNO— from 1 μ M H₂S based on the values reported by Cortese-Krott *et al.* (59). Thus, studies are warranted to resolve this conflict in the future. Nonetheless, all these species have been shown to possess anti-tumor effects by previous studies (46, 51, 255a), indicating the potential of a direct interaction between the two gaseous transmitters in cancer biology. Further studies are, therefore, needed to validate the interaction that may lead to the discovery of novel strategies for cancer therapy.

B. Crosstalk of H₂S with CO in cancer

The direct reaction between H₂S and CO has not yet been reported so far; however, emerging evidence suggests that the axis of CO–CBS–H₂S may be important in cancer regulation, which has been discussed next.

1. CO–CBS–H₂S axis in cancer regulation. CO is a gaso-transmitter generated from heme oxygenase (HO) by catalyzing the oxidative degradation of heme, a process highly relying on the availability of cellular O₂ (313, 319). So far, there is plenty of evidence suggesting that CO is an intrinsic modulator of H₂S biosynthesis by inhibiting CBS activity. For example, an increase of CO level in liver leads to the reduction of H₂S concentration and a subsequent increase of bile excretion, both of which are shown to be absent in CBS heterozygous knockout mice (255). In line with this, hypoxia increases CBS-mediated H₂S production through inhibition of HO-2 activity and CO biosynthesis, which may contribute to vasodilation of precapillary arterioles (190). It is worth mentioning that hypoxia can also lead to the suppression of H₂S oxidation, thereby augmenting H₂S signaling (210, 213), which could be very relevant to the development of solid tumors.

Moreover, the inhibitory effect of CO on CBS activity has been ascribed to its irreversible binding to the heme group of CBS (122, 234, 256). Currently, accumulative studies suggest that this regulation may be relevant to cancer development. As a tumor grows, it rapidly outgrows its blood supply, leading to a significantly lower concentration of O₂ in tumor tissues compared with that of normal tissues (104, 105). Such a low level of O₂ could lead to the reduction of CO biosynthesis and H₂S overproduction, which may be supportive for tumor growth through the beneficial effects of H₂S in cancer development as reviewed earlier. In accordance with this, the low enzymatic activity of HO-1 is found in moderately differentiated prostate tumors, which correlates with relatively worse clinical outcomes (303).

In addition, exposure to a low level of CO diminishes the rate of glycolysis in cancer cells and sensitizes them to chemotherapy with enhanced growth arrest and apoptosis (303), which are very similar to that after CBS inhibition (266, 267). Moreover, overexpression of HO-1 in cancer cells attenuates

FIG. 8. Direct interaction between H₂S and NO generates bioactive anti-cancer products. The interaction between H₂S and NO occurs even in physiological conditions and the key bioactive reaction products of the interaction are S/N-hybrid species, polysulfides, and HNO. Interestingly, all these species have been shown to possess anti-tumor effects by previous studies. HNO, nitroxyl; HSNO, thionitrous acid. Color images are available online.

FIG. 9. CO–CBS–H₂S axis in cancer regulation. In malignant solid tumors, hypoxia suppresses the activity of CO generating the enzyme HO, which leads to the reduction of CO level. This reduction can stimulate the activity of CBS and result in the upregulation of endogenous H₂S synthesis since CO serves as an intrinsic inhibitor of CBS. The increase of endogenous H₂S can be beneficial for cancer cell growth by promoting angiogenesis, activating anti-apoptotic pathways, and stimulating bioenergetic process. In addition, the activation of CBS itself is able to stimulate the methylation of PFKFB3, which enhances glycolysis and also contributes to bioenergetic stimulation. CO, carbon monoxide; HO, hemo oxygenase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bis-phosphatase 3. Color images are available online.

the growth of lung carcinoma xenografts in mice (203, 258, 277). However, it is notable that although the reduction of H₂S caused by CBS inhibition may be, at least partially, involved in this CO-mediated cancer inhibition, a direct demonstration of such involvement is still lacking.

Other than the decrease of H₂S, CBS inhibition by CO has also been found to cause demethylation of proteins such as 6-phosphofructo-2-kinase/fructose-2,6-bis-phosphatase 3 (PFKFB3) (325). The demethylation of PFKFB3 results in the attenuation of glycolysis in cancer cells (261). Therefore, it can be proposed that CBS activation on CO reduction in hypoxic conditions such as a tumor microenvironment may contribute to the maintenance of glycolysis partially by enhancing PFKFB3 methylation. The possible mechanisms underlying CO–CBS–H₂S axis in cancer regulation have been illustrated in Figure 9.

VI. The Potential of Targeting H₂S for Cancer Treatment

A. Treatment of cancer with H₂S biosynthesis inhibitors

All the three H₂S synthesizing enzymes, namely CSE, CBS, and 3MST, might be involved in cancer development, as shown in section III, however, only CBS has been demonstrated as a potential therapeutic target for cancer treatment, particularly in colon cancer (48, 102, 103, 266, 267) and ovarian cancer (24, 47). As a result, the discovery of CBS inhibitors has attracted more attention compared with their counterparts. In this section, we summarize the current CBS

inhibitors and their usage, particularly in cancer treatment. The chemical structures of these CBS inhibitors have been illustrated in Figure 10.

1. CBS inhibitor aminooxyacetic acid shows great potential in cancer therapy. Aminooxyacetic acid (AOAA) (Fig. 10a) is a traditional CBS inhibitor and has been extensively used in the field of H₂S biology. In colon cancer cell lines, AOAA treatment inhibits H₂S production, cell migration, and cell growth, closely mimicking the effect of CBS silencing (266, 267). A similar effect was observed in other cancer cell lines, including ovarian cancer cells (24), breast cancer cells (278), and pancreatic adenocarcinoma cells (259). *In vivo*, AOAA exhibits remarkable inhibition of HCT116 cell xenografts (267) and the proliferation of patient-derived colon tumor xenografts (102). Moreover, the inhibitory effect on breast cancer has also been shown in a xenograft mouse model (278). However, it is noticed that non-cancerous cells have not been included in most studies mentioned earlier. Therefore, it remains unclear whether AOAA has sufficient therapeutic window for cancer treatment.

Recently, Chao *et al.* (48) found that addition of a methyl ester group on AOAA enhances the cellular uptake of AOAA and the new molecule YD0171 (Fig. 10a) exerts higher potency on the growth inhibition of colon cancer cell line HCT116 both *in vitro* and *in vivo*. In addition, YD0171 reduces tumor growth in the patient-derived tumor xenograft model and causes no systemic toxicity in a 5-day safety study, indicating the value of the molecule for cancer treatment. On

FIG. 10. Pharmacological inhibitors of CBS. (a) Traditionally used CBS inhibitor and its derivative YD0171. Addition of a methyl ester group on AOAA enhanced the cellular uptake of AOAA, and the new molecule YD0171 exerted higher potency on the growth inhibition of colon cancer cells both *in vitro* and *in vivo*. The IC₅₀ was determined by Asimakopoulou *et al.* (9). (b) CBS inhibitors screened by Zhou *et al.* (348) from a chemical library containing 21,599 compounds. This is the first high-throughput screen with the effort to identify specific CBS inhibitors. (c) CBS inhibitors obtained from a 1900 chemical library by Thorson *et al.* (279). The two compounds suppress the activity of CBS without affecting that of CSE. (d) CBS inhibitors screened from a library of marine natural products (MICL-240 library) by Thorson *et al.* (279). (e) CBS inhibitor synthesized by McCune *et al.* (177). The inhibitor was synthesized based on the structure of the enzymatic product rather than the cofactor PLP, therefore it shows rather high specificity over other PLP-dependent enzymes. The IC₅₀ is not comparable among studies due to the differences in assay format. AOAA, aminooxyacetic acid; PLP, pyridoxal 5'-phosphate.

top of these, one should bear in mind that the selectivity of AOAA for CBS over CSE or other PLP-dependent enzymes is limited as it targets the PLP cofactor, which is commonly used by this class of enzyme (102). In line with this, a study has indicated that AOAA is three times less potent for CBS than CSE (9). Therefore, selective CBS inhibitors are of high demand.

2. Advances in the development of selective CBS inhibitors. To obtain novel selective CBS inhibitors, Zhou *et al.* (348) initiated the first high-throughput assay for the identification of potent and selective inhibitors of CBS in 2013. With a tandem-microwell assay, 21,599 agents from various libraries have been screened against recombinant human CBS (hCBS). As a result, 35 compounds have been confirmed as hits with an IC₅₀ of <50 μ M for the inhibition of hCBS. Among them, five compounds, namely MBSEW03275, JHU-8555/Quinaldine blue, MBS08407, NSC260610, and

NSC177365 (Fig. 10b), have shown a preference to inhibit hCBS over human CSE with MBS08407 and Quinaldine blue as the most selective ones. Interestingly, quinaldine blue is an anti-tumor drug approved by FDA; however, its molecular mechanism is not clear (75). This study implies that quinaldine blue may inhibit tumor growth by selectively suppressing the activity of CBS.

Later, Barrios and colleagues (279) conducted another screen of a small chemical library containing 1900 compounds with an H₂S probe-based assay. Eventually, two compounds, namely 1,4-naphthoquinone and tangeritin (Fig. 10c), were yielded that selectively suppress the activity of CBS without affecting that of CSE. Intriguingly, both the compounds have been suggested as potential anti-cancer molecules, especially for colon cancer in other studies (129, 191). Using a library of marine natural products (MICL-240 library), Barrios and colleagues (280) have recently performed another screen to identify novel CBS inhibitors. They

identified five hits ($IC_{50} < 150 \mu M$; Fig. 10d) as CBS inhibitors; however, the selectivity of these compounds remains elusive, as the effect of these compounds on CSE has not been tested. Nevertheless, these scaffolds may serve as useful starting points for the synthesis of selective CBS-targeted molecules in the future.

Apart from the earlier mentioned high-throughput strategy, McCune *et al.* (177) recently reported a novel way to synthesize selective CBS inhibitors. They chose the enzymatic product (L, L)-cystathionine, instead of the cofactor PLP, as a scaffold for the synthesis of inhibitor candidates. In the study, half of the inhibitor was constructed and then fused by olefin cross-metathesis. Among the inhibitors, CBSI 6S (Fig. 10e) exhibits the highest potency for CBS and reduces infarct volume in a rat transient middle cerebral artery occlusion model. Significantly, CBSI 6S shows 12-fold higher inhibition of CBS than that of gamma-aminobutyric acid aminotransferase, one of the best studied PLP dependent enzymes, indicating its good selectivity. Further studies are needed to test the effect of CBSI 6S on cancer cell proliferation.

B. Treatment of cancer with H_2S donors

H_2S donors with different properties have been developed and tested in various disease models. Herein, we review three categories of H_2S donors, including sulfide salts, phosphorodithioate derivatives, and allium sativum extracts and derivatives, with an emphasis on their application in cancer treatment (Fig. 11).

1. Sulfide salts. Sulfide salts (Fig. 11a), namely NaSH and Na_2S , are the most commonly used H_2S donors in the field of H_2S biology. They readily dissolve in physiological solution buffers and water and form HS^- and H_2S (157). As they do not have any backbone structures, it is believed that the biological effects derived from these molecules are solely from H_2S . In the context of cancer biology, the effect of salt sulfides appears to be concentration dependent. Specifically, it promotes cancer cell proliferation at low doses and inhibits cancer cell growth at high concentrations (102). Perhaps due to the distinct effects caused by concentrations, no studies have been performed to show their efficacy for cancer inhibition within *in vivo* models. In addition, the concentration-dependent effect of these H_2S donors has also been observed in non-cancerous cells such as heart cells and smooth muscle cells (15), indicating a possible marginal therapeutic index of these compounds in cancer treatment. The other concern of utilizing sulfide salts is that they release H_2S at an uncontrolled manner and may cause acute toxicity to organs.

Taken together, sulfide salts serve as great tool compounds to illustrate H_2S -mediated biological effects and the underlying mechanisms whereas the feasibility is rather low to translate them as a therapeutic drug, especially for cancer treatment.

2. Phosphorodithioate derivatives. GYY4137 (Fig. 11b) is the first phosphinodithioate-derived H_2S -releasing compound that was synthesized in 2008 (162). It slowly releases H_2S in aqueous solution *via* a two-step process and after intravenous or intraperitoneal injection in animals, closely

FIG. 11. Structural components of H_2S donors with anti-tumor activity. (a) Chemical structure of sulfide salts, including sodium hydrosulfide and sodium sulfide. (b) Chemical structure of phosphorodithioate derivatives donating H_2S . FW1256, derivative of GYY4137, has a higher H_2S -releasing rate and higher potency of anti-tumor activity compared with GYY4137. (c) Chemical structure of *Allium sativum* extracts and derivatives, including Allicin, DAS, DADS, and DATS. DADS, diallyl disulfide; DAS, diallyl sulfide; DATS, diallyl trisulfide.

mirroring the physiological releasing manner of H₂S from mammalian cells (5, 162). Therefore, it has been extensively used to study both physiological and therapeutic effects of H₂S. The anti-cancer effect of GYY4137 has been demonstrated both *in vitro* and *in vivo* (149, 150, 162, 171). Importantly, GYY4137 induces cell apoptosis in cancer cell lines without affecting the cell viability of noncancerous fibroblast cells (149, 150), indicating the potential value of the molecule for cancer treatment. However, examination of published data (149, 150) clearly indicates that the concentrations and/or doses of GYY4137 employed to exert the anti-cancer effect are high (400–1000 μ M for *in vitro*; 100–300 mg/kg for *in vivo*), indicating its marginal potency. However, H₂S production from GYY4137 is inefficient (5), and very little H₂S is produced, necessitating its use at high concentrations/doses.

To improve the anti-cancer potency of GYY4137, Feng *et al.* (76) have recently synthesized a series of compounds based on the structure of GYY4137. Among these compounds, FW1256 (Fig. 11b) has shown superior potency in MCF7 tumor spheroids compared with GYY4137 (IC₅₀: 5.7 μ M for FW1256 vs. 368 μ M for GYY4137) and caused cancer cell death through apoptosis by activating PARP and caspase-7. At a concentration inhibiting cancer cell proliferation, no toxic effect is observed in noncancerous fibroblast cell Wi-38. Further analysis with a series of these compounds shows a positive correlation between the anti-cancer potency and H₂S-releasing rate, which may provide a guide for the future development of such compounds. However, the study did not show the anti-tumor activity of FW1256 *in vivo*, which needs to be done in the future.

3. *Allium sativum* extracts and derivatives. *Allium sativum* (garlic) has been extensively used for numerous diseases, including cardiovascular disease and cancer in traditional Chinese medicine (324). It is abundant with sulfur-containing compounds that can be easily recognized with a smell. Among these compounds, allicin (diallyl thiosulfinate; Fig. 11c) has been extracted and studied thoroughly. Intriguingly, compiling evidence suggests that allicin possesses anticancer effects in various types of cancer both *in vitro* and *in vivo* (17, 218, 273). For instance, allicin significantly inhibits cell proliferation of several colon cancer cell lines such as Caco-2, HT-29, and HCT116 (17).

In addition, allicin administration apparently inhibits tumor growth in L5178Y lymphoma-bearing mice (218). Allicin is very unstable and decomposes quickly into several compounds, including diallyl sulfide, diallyl disulfide, diallyl trisulfide, and allyl methyl sulfide (Fig. 11c). Therefore, these individual compounds have also been synthesized and tested in various disease models. The results have shown that the number of sulfur atoms well reflects the efficacy of these molecules not only in cancer inhibition (192) but also in vasodilation (22), which positively correlates with the H₂S-releasing capacity. In spite of this, further studies are still needed to investigate whether the effects are derived from other reactive sulfur species or H₂S directly, as suggested by a recent study (61).

In addition, SPRC (Fig. 11c), a novel stimulator of endogenous H₂S, is a structural analogue of S-allylcysteine that is derived from garlic component S-alkyl cysteine sulfoxides. It has been shown to stimulate H₂S production continuously

in mammalian cells (295). Recently, SPRC was found to cause cell cycle arrest at the G1/S phase in gastric cancer cell line SGC-7901 and subsequent apoptosis both *in vitro* and *in vivo* (123). Intriguingly, the anti-cancer effect of SPRC can be blocked by CSE inhibition (123), indicating that the effect can be at least partially attributed to the production of H₂S. However, the marginal potency (IC₅₀ $\approx$ 5 mM) may be an issue to use this compound as cancer therapy, which needs to be improved in the future.

4. H₂S-releasing hybrids

a. H₂S-releasing non-steroid anti-inflammatory drugs

(1) H₂S-releasing non-steroid anti-inflammatory drugs possess anti-cancer activity. The finding that H₂S is able to enhance ulcer healing (291) has impelled the synthesis of H₂S-releasing non-steroid anti-inflammatory drugs (HS-NSAIDs) to limit NSAIDs caused gastrointestinal side effect. Usually, 5-(4-hydroxyphenyl)-3H-1, 2-dithiole-3-thione (ADT-OH) is covalently conjugated with an NSAID to form a hybrid (128). So far, a number of HS-NSAIDs have been developed, including HS-Sulindac, HS-Ibuprofen, HS-Mesalamine, HS-Naproxen, HS-Diclofenac, HS-Aspirin (HS-ASA), and HS-Indomethacin (127). Most of these drugs have shown less damage to the gastrointestinal tract compared with their respective NSAID (127), which favors the initial hypothesis. Besides inflammation, NSAIDs, in general, also serve as potential chemopreventive agents against cancer as indicated by Kune *et al.* (144) that subjects taking NSAIDs have a significantly lower incidence of colon cancer.

Recently, four HS-NSAIDs, including HS-Sulindac, HS-Ibuprofen, HS-naproxen, and HS-ASA (Fig. 12), have been tested within 11 cancer cell lines from six tissue origins across from solid tumor to leukemia (50). Remarkably, all the HS-NSAIDs tested exert inhibitory effects on the growth of the 11 cancer cell lines. To achieve the same extent inhibition, the parental NSAID treatment alone requires 38- to 1300-fold higher concentration (50), indicating a synergic effect of H₂S and NSAID on cancer inhibition. The anti-tumor effect of HS-NSAIDs was further demonstrated in several *in vivo* models (50). Taken together, HS-NSAIDs not only increase the therapeutic index but also enhance the anti-tumor activity of NSAIDs, showing their potential value as novel anti-cancer agents. Nonetheless, though existing data showed that some H₂S-NSAIDs such as HS-Diclofenac can release H₂S in plasma and their effects can be mimicked by an injection of ADT-OH and corresponding NSAID (160), however, whether these compounds release H₂S and NSAIDs simultaneously that account for the subsequent anti-cancer effect was not established in many cases.

(2) Mechanisms of action of H₂S-NSAIDs in cancer inhibition. Along with the recognition of the remarkably anti-cancer potential of HS-NSAIDs, the mechanisms underlying such effects have also been thoroughly studied, which has been summarized in Figure 13. Chattopadhyay *et al.* (50) have found that the four HS-NSAIDs (HS-Sulindac, HS-Ibuprofen, HS-naproxen, and HS-ASA) induce apoptosis in HT-29 colon cancer cells accompanied by cell cycle arrest in the G0/G1 phase, which has been further paralleled in breast cancer MDA MB-231 (98). In addition, also using MDA-MB 231 cells, it is suggested by studies from Kashfi *et al.* that HS-

FIG. 12. Structural components of H₂S-releasing NSAIDs with anti-tumor activity. ADT-OH is covalently conjugated with an NSAID to form a hybrid that releases H₂S and NSAID simultaneously. The traditional NSAIDs are shown in the shaded box. ADT-OH, 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione; NSAID, non-steroid anti-inflammatory drug. Color images are available online.

ASA may inhibit cancer cell growth by suppression of the NF- κ B signaling pathway *via* preventing the phosphorylation of the I κ B kinases (12, 49). Moreover, it has been shown that HS-ASA concentration dependently results in the cellular ROS formation, which may also contribute to the following cancer cell apoptosis (50, 54). Though the mechanisms underlying HS-ASA-mediated ROS production remain elusive, it is worth mentioning that H₂S, in fact, can behave as a pro-

oxidant through the interaction with dioxygen to produce sulfur-centered and oxygen-centered free radicals (10, 260). This could induce a redox shift over the redox set points of cancer cells (*i.e.*, redlines) and result in cancer cell death (232).

Intriguingly, the suppression of the wnt/ β -catenin pathway is suggested to be the mechanism underlying the anti-cancer effect of HS-ASA in T cell acute lymphoblastic leukemia (52, 88). Other than these, modulation of thioredoxin/thioredoxin

FIG. 13. Mechanisms of action of HS-NSAIDs in cancer inhibition. Multiple mechanisms have been suggested to underlie the anti-cancer effect of HS-NSAIDs. These mechanisms include, but are not limited to, cell cycle arrest, Suppression of the NF- κ B signaling pathway, stimulation of ROS formation, suppression of the wnt/ β -catenin pathway, modulation of thioredoxin/thioredoxin reductase-1, and modulation of xenobiotic-metabolizing enzymes. HS-NSAID, H₂S-releasing non-steroid anti-inflammatory drug; ROS, reactive oxygen species. Color images are available online.

FIG. 14. The chemical structures of NOSH compounds possessing anti-cancer activity. The mother compound aspirin is shaded in gray; H₂S-releasing moiety is shaded in pink; and NO-releasing moiety is green. NOSH-1, (4-(3-thioxo-3H-1, 2-dithiol-5-yl) phenyl 2-((4-(nitrooxy)butanoyl)oxy)benzoate); NOSH-2, (4-(nitrooxy)butyl 2-((4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy)carbonyl)phenyl)); NOSH-3, (4-carbamothioylphenyl 2-((4-(nitrooxy)butanoyl)oxy)benzoate); NOSH-4, (4-(nitrooxy)butyl 2-(5-((R)-1,2-dithiolan-3-yl)pentanoyloxy)benzoate). Color images are available online.

reductase-1 and/or xenobiotic-metabolizing enzymes may also contribute to the anti-cancer effect of HS-NSAIDs, as suggested by various studies (16, 50). As clearly seen from what has been described earlier, HS-NSAIDs exert their anti-cancer effect through multiple mechanisms. Nevertheless, why HS-NSAIDs possess anti-cancer activity with such a higher potency than their parental drugs is not yet revealed.

Therefore, it would be better to compare the efficacy and mechanism of action among H₂S donor, NSAID, H₂S donor together with NSAID, and HS-NSAID hybrid to obtain a better understanding of the possible interactions between H₂S and NSAIDs in the treatment of cancer.

b. NOSH compounds as anti-cancer agents. Due to the anti-inflammatory effects of exogenous NO and H₂S, it is hypothesized that compounds simultaneously donating NO and H₂S may achieve higher potency in the suppression of inflammation. This class of compounds is termed NOSH compounds (51, 138). Currently, four such NOSH compounds have been reported, including NOSH-1 [(4-(3-thioxo-3H-1,2-dithiol-5-yl) phenyl 2-((4-(nitrooxy)butanoyl)oxy)benzoate)], NOSH-2 [(4-(nitrooxy)butyl 2-((4-(3-thioxo-3H-1, 2-dithiol-5-yl)phenoxy) carbonyl)phenyl)], NOSH-3 [(4-carbamothioylphenyl 2-((4-(nitrooxy)butanoyl)oxy)benzoate)], and NOSH-4 [(4-(nitrooxy) butyl 2-5-((R)-1,2-dithiolan-3-yl)pentanoyloxy)benzoate)] (Fig. 14), all of which use aspirin as a scaffold coupled with NO and H₂S-releasing moieties (138). As expected, these compounds show great potential in inflammatory inhibition (128, 147, 235). Remarkably, all of the four NOSH compounds exhibit

extreme effectiveness in the growth inhibition of cancer cells, including colon cancer (HT-29, HCT15, and SW480), breast cancer (MCF7, MDA MD-231, and SKBR3), T cell leukemia (Jurkat), pancreatic cancer (BxPC3 and MIAPaCa-2), prostate cancer (LNCaP), and lung cancer (A549) (138). Among these compounds, NOSH-1 was shown to be the most potent one with IC₅₀ ranging from 48 to 240 nM for inhibition of cancer cell growth at 24 h (138).

Compared with its parental ASA, the potency for inhibition of cancer cell growth is increased by 100,000-fold, 60,000-fold, 600-fold, and 16,000-fold for NOSH-1, -2, -3, and -4, respectively, in HT-29 colon cancer cells (138). Significantly, the anti-tumor effect of NOSH-1 has been demonstrated in a human colon cancer xenograft model, which shows an 85% reduction in tumor volume after treatment for 18 days (51). Although the underlying mechanisms of NOSH compounds have not well understood yet, it is highly likely that they may include the aforementioned mechanisms of HS-NSAIDs and also the interaction between NO and H₂S, which needs to be tested in the future.

C. The therapeutic potential of H₂S donation for cisplatin nephrotoxicity

Cisplatin is a widely used chemotherapeutic drug for cancer treatment (335). Due to the remarkable ability to treat various forms of cancers, it has gained the reputation as “the penicillin of cancer” (320). However, its effective usage for cancer therapy has been largely limited because of numerous

adverse effects (81). Among these adverse effects, nephrotoxicity is the most prevalent one as evidence shows that more than 30% of patients show symptoms of acute kidney injury after the administration of cisplatin (239). This causes either discontinuation of treatment or severe renal damage. After years of efforts, some success has been made to curtail cisplatin nephrotoxicity. For instance, hydration of patients during cisplatin administration appears to reduce the occurrence after renal dysfunction (11, 57). Despite this, cisplatin nephrotoxicity remains a crucial factor compromising the use and efficacy of cisplatin in the therapy of cancer.

Very different from traditional chemotherapeutic drugs, H₂S exhibits both cell protective effects and anti-cancer effects. Interestingly, H₂S donation with GYY4137 inhibits cancer cell growth at doses that exhibit protective effects against oxidative stress and/or inflammation-induced cell injuries (57, 150, 155, 304). Therefore, we proposed that H₂S donation may be a promising strategy for the treatment of cisplatin nephrotoxicity, which is mainly caused by oxidative stress and inflammation in renal proximal tubule cells (8, 217). By this strategy, we speculate that H₂S donation may alleviate cisplatin caused renal toxicity and add on more anti-cancer activity when combining with cisplatin in cancer cells. This hypothesis is partially supported by recent proof-of-concept studies that NaSH attenuates cisplatin caused renal toxicity in rat models (3, 67) whereas whether NaSH influences the anti-cancer activity of cisplatin is not studied.

However, a recent report from Liu *et al.* somewhat disfavors the hypothesis because they have shown that H₂S slowly releasing donor GYY4137 aggravates cisplatin-induced renal damage by increasing inflammatory response (167). But several defects in using GYY4137 can be identified after clear examination of the study, such as (i) GYY4137 is prepared and stored in dimethyl sulfoxide, which has been shown to be detrimental and to accelerate GYY4137 decomposition (162, 178, 179); (ii) the study used a rather low dose of GYY4137 (21 mg/kg). It is likely that H₂S might be not provided adequately considering the releasing property of H₂S by the compound (178, 179); (iii) the chemical analogue of GYY4137, ZYJ1122 (150), was not used in the study, thus the possibility that the chemical backbone of GYY4137 molecule aggravated cisplatin nephrotoxicity cannot be ruled out, particularly at such a low concentration of generated H₂S. As such, further studies are warranted to unclothe the effects of H₂S slowly releasing compounds such as GYY4137 on both cisplatin caused renal toxicity and the anti-cancer activity of cisplatin.

Besides, as discussed earlier, the recently developed H₂S-releasing hybrids such as H₂S-NSAIDs and NOSH-NSAIDs possess similar effects to those of H₂S slowly releasing compounds but with greater potency not only in cellular protection but also in cancer inhibition. Therefore, they may have promising potential in the treatment of cisplatin nephrotoxicity while without influencing its anti-cancer activity even if not potentiating it.

VII. The Novelty and Challenges of H₂S-Based Cancer Therapy

A. The novelty of H₂S-based cancer therapy

Cancer is one of the main threats to human beings and tremendous efforts have been invested in the treatment of this

devastating disease. Traditionally, the discovery of anti-cancer drugs was led by the identification of cytotoxic compounds. For several decades, these drugs similar to alkylating agents and nitrogen mustard have shown effectiveness against various types of tumors. However, they also induce severe adverse effects because they can hardly discriminate cancer cells and normal cells.

The recent advances in molecular sciences and genomics have recognized the genetic basis of tumorigenesis and generated potential new targets. This has led to a change in the paradigm of anticancer drug discovery toward molecularly targeted therapy (27). The paradigm shows success as indicated by the emergence of molecular targeted drugs, including imatinib, gefitinib, and bortezomib. However, several limitations appear in recent years, such as (i) cancer cells can develop resistance to these drugs; (ii) the treatment can be lost as sometimes the target changes; (iii) drugs may be difficult to be developed for some targets; (iv) one drug can hardly abolish tumor growth due to the heterogeneity of tumor populations; and (v) the drug may be not able to adequately penetrate into solid tumors. This is well reflected by the emergence of a changing paradigm by targeting multiple targets using either drug cocktails or multiple-targeted drugs, particularly for complex diseases such as cancer, diabetes, acquired immune deficiency syndrome, *etc.* (58, 288).

H₂S is a potential therapeutic agent with multiple biological targets with unique properties. In contrast to toxic chemotherapeutic drugs, H₂S possesses protective effects in multiple organs even in the doses that are able to inhibit tumor growth as shown with the slowly releasing donor GYY4137 (27, 161, 178, 179). Thus, it is likely that the existence of H₂S is critical for the maintenance of cellular homeostasis, including both normal cells and cancer cells. This is supported by the evidence that H₂S is essential to regulate the homeostasis of redox (322) and thiols (91). Thus, modulation of H₂S may disrupt the cellular homeostasis of cancer as a whole, thereby leading to subsequent death. Perhaps due to this, H₂S-based therapy has been demonstrated to be effective in multiple cancer types (91, 149, 150).

Importantly, the distinct level of endogenous H₂S in cancerous and non-cancerous cells and some other reasons might afford them the different tolerances to H₂S supplementation or H₂S inhibition. This has been indicated by the demonstrated therapeutic window of the H₂S-based approach for cancer therapy (149, 150). In addition, H₂S is a small lipophilic molecule that allows it to freely penetrate into the cell membrane of all types and become biologically active (37). This may at least have two implications: (i) H₂S may have a significant influence on the tumor microenvironment, which has shown to be important for tumor progression (283); (ii) it may be able to readily infiltrate into solid tumors. Compared with molecularly targeted therapy, H₂S is shown to affect multiple targets in cancer cells and as a result may overcome the limitations of molecular targeted drugs mentioned earlier. Therefore, although still in its infancy, H₂S-based therapy may represent a novel and unique strategy for cancer treatment.

B. The challenges of H₂S-based cancer therapy

Despite the earlier mentioned merits, successful translation of H₂S as a therapeutic agent still faces great challenges,

particularly for cancer therapy. It is no doubt that H₂S-related toxicity is always the first obstacle in the development of H₂S-based drugs. Notably, promising progress has been made in the field as clearly seen from the successful transition of H₂S donors such as SG-1002 and ABT-346 from phase I into phase II clinical trials (292). However, to achieve the cancer killing effect, higher doses of H₂S donors are expected compared with those for other indications such as heart failure and inflammatory diseases. This may narrow the therapeutic window of these H₂S donors in the context of cancer therapy, which could be an impediment for their further development.

In addition, most studies, particularly those done with H₂S acute releaser NaSH or Na₂S, seemed to neglect the toxic effect of these compounds themselves and failed to include normal cells to profile the therapeutic window of H₂S donors when studying their anti-cancer activities (103, 317, 318, 330, 338). Therefore, the demonstration of whether and how H₂S discriminates cancer cells and normal cells will surely encourage the enthusiasm to develop H₂S donors for cancer therapy.

VIII. Future Directions

A. Relationship between H₂S level and cancer progression

Although evidence suggests the implication of H₂S in cancer development, a clear relationship between H₂S level and cancer progression remains lacking. For instance, no reports have shown the level of H₂S in the plasma or tumor microenvironment in cancer patients compared with normal subjects till now. Moreover, most studies have ignored the fact that modulation of H₂S-producing enzymes can alter not only the level of H₂S but also other byproducts such as pyruvate, glutathione, and L-homocysteine (169). The possibility that the altered levels of these byproducts have influenced the cell viability of cancer cells has not been excluded in previous studies. In addition, the levels of H₂S have not been measured on modulation of H₂S-producing enzymes as well as in most studies. Therefore, a clear relationship between H₂S level and cancer progression may be beneficial to decipher the role of H₂S in cancer development, thereby helping develop better H₂S-based small molecules for cancer therapy.

B. Test of drug-like H₂S donors in cancer

H₂S donors such as GYY4137 and SPRC have shown promising anti-tumor effects. However, their marginal potency and as-yet-unknown human toxicity may hinder their use as anti-cancer agents in humans. Recently, a batch of drug-like H₂S donors have been developed and tested to be safe in clinical trials. Among them, an orally active H₂S donor SG-1002 is undergoing Phase II study for heart failure; ATB-346 is being tested for osteoarthritis; ATB-352 is under the investigation for acute pain; and ATB-350 is being evaluated for thrombosis (292). It is worth mentioning that SG-1002 has been demonstrated to be safe and produced a sustained increase in both plasma H₂S and plasma NO levels in humans (230). Interestingly, a promising outcome has been observed for some pediatric cancer patients as described in the patent (patent number: US8771755B2). The detailed information of such H₂S donors can be found in Wallace and Wang's recent review (292). Testing of these drug-like H₂S

donors will not only consolidate the anti-cancer effect of H₂S donation but also shed light on the translation of H₂S as a therapeutic agent for human cancers.

C. Understand the molecular mechanisms underlying H₂S effects

H₂S elicits most of its biological effects by induction of protein persulfidation. Among these target proteins, NF- κ B and Keap1/Nrf2 are closely related to cancer progression. Interestingly, endogenous H₂S or H₂S at low levels has been found to stimulate the translocation of p65/p50 into the nucleus (245) whereas high levels of H₂S apparently inhibit the activity of NF- κ B, particularly at inflammatory conditions (92, 309). This may partially explain the dual role of H₂S in cancer development. The revelation and in-depth depiction about the interaction between H₂S and its target proteins will surely facilitate our understanding on the role of H₂S in cancer biology. On the other hand, protein cysteine persulfides may be generated by CSE in an H₂S-independent manner (112), implying that CSE may modulate cancer cell survival even in the absence of changes of H₂S levels in cancer cells.

D. Confirm H₂S-linked persulfidation of target proteins

As reviewed earlier, several target proteins have been identified to be persulfidated and, therefore, take part in cancer development. However, the reliability of the most commonly method for the identification, namely the biotin switch assay was recently questioned because it seems that the thiol-blocking reagent S-methyl methanethiosulfonate used in the method would not differentiate free thiols and persulfides (78). Therefore, further studies are warranted to confirm H₂S-linked persulfidation of target proteins by employing a more reliable method. Recently, two other persulfide detection methods, namely tag switch assay and protein persulfide detection protocol (ProPerDP) were developed by Xian and colleagues (337) and Nagy and colleagues (66), respectively. Significantly, the ProPerDP method is to date the only method alkylating sulfhydryl group before cell lysis that potentially avoids artificial redox post-translational modifications, including persulfidation, during lysing cells (66), representing a huge merit of this method.

E. Crosstalk of H₂S with NO in cancer

For a long period, H₂S and NO signaling pathways have been observed to be intimately intertwined with mutual potentiation of responses, particularly in the cardiovascular system (198). However, the crosstalk between these two gaseous transmitters remains largely unexplored in the context of cancer biology. An article from Cortese-Krott *et al.* has recently identified the key bioactive reaction products of NO/H₂S interaction to be S/N-hybrid species, inorganic polysulfides, and nitroxyl in physiological conditions (59). Intriguingly, all these species have been shown to possess anti-tumor effects by previous studies (46, 51, 255a), indicating the potential of the crosstalk between the two gaseous transmitters in cancer biology. However, the validation of such interaction is rarely studied in the field of cancer biology. It is expected that an in-depth understanding of such interaction may not only facilitate our understanding on tumorigenesis but also lead to novel strategies for cancer treatment.

F. Inorganic polysulfide accounts for the anti-cancer effect of H₂S?

H₂S is a very short-lived molecule; however, numerous studies have shown the long-lasting biological effects of H₂S in mammalian systems. Therefore, it is reasonable to speculate whether the formation of certain more stable substance(s) accounts for H₂S-derived effects. Kimura and colleagues have recently identified inorganic polysulfides in the solution of NaSH (114). Intriguingly, compared with NaSH, the polysulfides (Na₂S₂, Na₂S₃, and Na₂S₄) elicit much stronger effects on the activation of TRPA1 in rat astrocytes (214) and activation of Keap1/Nrf2 in neuronal cells (40, 214). The existence of endogenous inorganic polysulfides has also been demonstrated by the group (136). Moreover, garlic-derived polysulfides have long been well known to possess anti-tumor effects (192). Therefore, it will be of great value to evaluate whether inorganic polysulfides are the active molecules derived from H₂S and display the anti-tumor effect.

G. A new regulatory circuit of thioredoxin and H₂S by controlling persulfidation in cancer?

Thioredoxin, a small redox-regulating protein, has been found to be highly expressed in multiple cancers and plays important roles in cancer cell survival (126). Notably, two separate studies recently showed that thioredoxin acts as depersulfidase and, therefore, may control H₂S signaling by reducing protein persulfidation and regenerating H₂S (66, 302). Interestingly, H₂S was known to upregulate the expression of thioredoxin by activating the Keap 1–Nrf2 pathway (38). Moreover, the fourth interaction mediated by H₂S was shown to reduce the disulfide bonds of proteins (220, 289), coincidentally, an event leading to thioredoxin activation (126), suggesting a possibility of direct interaction between H₂S and thioredoxin. Altogether, this evidence implies a likelihood that thioredoxin and H₂S may work coordinately to control persulfidation in cancer cells and therefore their survival and progression.

H. H₂S-mediated immune cell regulation in cancer progression and therapy

The crosstalk between immune cells and cancer cells has gained great attention due to the impressive clinical benefits of restoration of tumor-suppressed immune function. Currently, it has been shown that H₂S can regulate the physiological function of immune cells. For example, Roberts and colleagues (183, 184) found that H₂S is an endogenous potentiator of T cell activation. Recently, Yang *et al.* (330) showed that H₂S deficiency by knocking out CBS led to systemic autoimmune disease due to regulatory T (Treg) cell deficiency, suggesting that H₂S takes part in the regulation of Treg-cell-associated immune homeostasis. Despite this, the studies of H₂S-mediated immune cell regulation remain lacking in the context of cancer progression and therapy.

IX. Concluding Remarks

On recognition to be the third gaseous transmitter, the image of H₂S is expanding in mammalian systems. In the past decade, numerous studies demonstrated complicated roles of H₂S in the context of cancer development. Based on the

published evidence, bell-shaped models have been proposed to explain the role of H₂S in cancer development. Specifically, endogenous H₂S or a relatively low level of exogenous H₂S may exhibit pro-cancer effects, whereas exposure to H₂S for long period or a high amount may lead to cancer cell death. However, one should bear in mind that the consequence of CSE or 3MST inhibition in cancer cell is still largely unexplored, which needs to be examined in the future. Nevertheless, the models, indeed, have been favored by most studies in the field.

Remarkably, H₂S is not alone in the regulation of cancer biology, because it intertwines with the other two gasotransmitters such as NO and CO. Better portrayal of the crosstalk among these gaseous transmitters may not only lead to an in-depth understanding of cancer progression but also shed light on novel strategies for cancer therapy. Recently, the developments of CBS inhibitors and H₂S donors have gained enthusiasm because of their potential value as novel anti-cancer therapeutics. More selective CBS inhibitors compared with traditionally used AOAA are now available and some of them have shown anti-tumor activity. Moreover, H₂S-releasing hybrids, especially HS-NSAIDs and HSNO compounds, have shown very promising anti-tumor activity with marginal toxicity, indicating their value for cancer therapy. Nonetheless, the development and application of H₂S-based therapy for cancer treatment is still in its infancy. In the future, more studies are still warranted to identify H₂S-targeted signaling molecules and develop better compounds with higher selectivity and potency.

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Abbreviations Used

3MP = 3-mercaptopyruvate
3MST = 3-mercaptopyruvate sulfurtransferase
ADT-OH = 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione
AE = anion exchanger
AKT = protein kinase B
AOAA = aminooxyacetic acid
ATP = adenosine triphosphate
B-TECs = endothelial cells obtained from breast carcinomas
CAT = cysteine aminotransferase
CBS = cystathionine β -synthase
CO = carbon monoxide
CSE = cystathionine γ -lyase
DAO = D-amino acid oxidase

Abbreviations Used (Cont.)

eNOS = endothelial nitric oxide synthase
 ERK = extracellular signal-regulated kinase
 GC = gas chromatography
 GluT = glucose transporter
 H₂S = hydrogen sulfide
 hCBS = human CBS
 HIF-1 α = hypoxia inducible factor 1 α
 HNO = nitroxyl
 HO = hemo oxygenase
 HPLC = high-performance liquid chromatography
 HS-ASA = HS-aspirin
 HSNO = thionitrous acid
 HS-NSAID = H₂S-releasing non-steroid
 anti-inflammatory drug
 K_{ATP} = ATP-sensitive potassium
 MBB = monobromobimane
 MEK1 = ERK activator kinase 1
 Na₂S = sodium sulfide
 NADH = nicotinamide adenine dinucleotide
 NADPH = nicotinamide adenine dinucleotide
 phosphate
 NaSH = sodium hydrosulfide
 NF- κ B = nuclear factor kappa-light-chain
 enhancer of activated B cells
 NHE = sodium/proton exchanger
 NO = nitric oxide

NOSH-1 = (4-(3-thioxo-3H-1, 2-dithiol-5-yl)
 phenyl 2-((4-(nitrooxy)butanoyl)oxy)
 benzoate)
 NOSH-2 = (4-(nitrooxy)butyl (2-((4-(3-thioxo-3H-1,
 2-dithiol-5-yl)phenoxy)carbonyl)phenyl))
 NOSH-3 = (4-carbamothioylphenyl
 2-((4-(nitrooxy)butanoyl)oxy)benzoate)
 NOSH-4 = (4-(nitrooxy)butyl 2-(5-((R)-1,2-
 dithiolan-3-yl)pentanoyloxy)benzoate)
 Nrf2 = transcription factor NF-E2-related
 nuclear factor 2
 ProPerDP = protein persulfide detection protocol
 PARP1 = poly [ADP-ribose] polymerase 1
 PFKFB3 = 6-phosphofructo-2-kinase/fructose-2,
 6-bis-phosphatase 3
 PI3K = phosphoinositide-3-kinase
 PLP = pyridoxal 5'-phosphate
 RNS = reactive nitrogen species
 ROS = reactive oxygen species
 SAM = S-adenosyl-L-methionine
 SPRC = S-proargyl-cysteine
 SQOR = sulfide quinone oxidoreductase
 STAT3 = signal transducer and activator
 of transcription 3
 TNF = tumor necrosis factor
 TRAF = TNFR-associated factor
 VEGF = vascular endothelial growth factor