Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter

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Abstract | Hydrogen sulfide (H_2S) has become recognized as an important signalling molecule throughout the body, contributing to many physiological and pathological processes. In recent years, improved methods for measuring H_2S levels and the availability of a wider range of H_2S donors and more selective inhibitors of H_2S synthesis have helped to more accurately identify the many biological effects of this highly reactive gaseous mediator. Animal studies of several H_2S-releasing drugs have demonstrated considerable promise for the safe treatment of a wide range of disorders. Several such drugs are now in clinical trials.

Cystathionine γ-lyase (CSE). An enzyme that converts L-cysteine into hydrogen sulfide, pyruvate and ammonia. It requires the cofactor pyridoxal phosphate (vitamin B6) for this activity.

Cystathionine β-synthase (CBS). An enzyme that catalyses the conversion of homocysteine to cystathionine (the first step in the trans-sulfuration pathway) and the condensation of homocysteine and cysteine to form cystathionine and hydrogen sulfide.

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Over the past 15 years, hydrogen sulfide (H_2S) has become recognized as a crucial signalling molecule with a wide range of physiological functions[1,2]. It can profoundly affect most organ systems in animals and humans, but it also contributes to many functions in plants and prokaryotes. Indeed, H_2S had important roles in the development of life on Earth during the 500 million years before photosynthesis[3]. The realization of the biological importance of H_2S in numerous cells, tissues and organs is now shedding light on the pathogenesis of various human diseases, and paving the way for innovative therapeutic interventions.

In this article, we review the pathways for the synthesis and metabolism of H_2S (FIG. 1), its major mechanisms of action, and some of the key disease processes in which H_2S appears to participate, as well as the tissues affected. Methods for measuring H_2S levels *in vivo*, which are crucial for the study of H_2S biology, are discussed in BOX 1. Also discussed are the concerns associated with the reliability and accuracy of such measurements, which have triggered debate on the physiologically relevant endogenous levels of H_2S and the use of H_2S donors in high micromolar concentrations to mimic the physiological actions of H_2S. Finally, we provide examples of attempts to exploit the actions of H_2S in the design of novel therapeutic agents for the treatment of arthritis, inflammatory bowel disease, myocardial dysfunction and chemoprevention of cancer.

H_2S synthesis and metabolism

H_2S synthesis. H_2S is produced in mammalian cells mostly through the reverse trans-sulfuration pathway (FIG. 1). Two pyridoxal 5′-phosphate (PLP)–dependent enzymes — cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) — catalyse the production of H_2S, ammonia and pyruvate from L-cysteine and homocysteine, respectively. A PLP-binding domain is common to CSE and CBS, and is crucial for their catalytic activities. Unlike CSE, CBS contains a 70-amino-acid haem domain in its amino terminus, which equips CBS to perform three functions. First, it offers an interaction site for two other gasotransmitters: nitric oxide (NO) and carbon monoxide. Second, it can act as a redox sensor to regulate its own production of H_2S (notably, without a haem domain, CBS is no longer reactive to oxidative stress)[4]. Third, it performs as an oxygen sensor for the regulation of CBS degradation: an increase in the partial pressure of oxygen leads to the oxygenation of the haem group such that the conformational change of CBS can be recognized by Lon protease, triggering its degradation of CBS[5].

Like CSE and CBS, cysteine aminotransferase (CAT) uses PLP as a cofactor and converts cysteine to 3-mercaptopyruvate. Using zinc as a cofactor, 3-mercaptopyruvate sulfurtransferase (MST) transfers the sulfur in the sulfane group of 3-mercaptopyruvate to other sulfur acceptors. In essence, MST acts as a sulfur carrier, rather than an H_2S producer, as the sequential reactions that are catalysed by CAT and MST generate sulfane sulfur. This bound sulfur has to be released or reduced to liberate H_2S (REF. 6). Another sulfur-carrying enzyme in our bodies is rhodanese (also known as thiosulfate sulfurtransferase)[7]. However, the biological and physiological importance of this enzyme in endogenous H_2S metabolism has not been fully determined. In contrast to MST, which is localized both in the cytosol and mitochondria, rhodanese is a true mitochondrial protein.

H_2S metabolism and excretion. Unlike NO, H_2S is relatively stable in body fluids. In the circulation and in the cytoplasm, free H_2S can be scavenged by methaemoglobin[8] or by metallo- or disulfide-containing macromolecules that function as sulfane-sulfur and bound-sulfate pools. Oxidation and methylation are another two mechanisms of H_2S metabolism. H_2S is oxidized sequentially in the mitochondrion to thiosulfate and then to sulfite, with the end-product under physiological conditions being sulfate[9]. The mitochondrial enzyme sulfide-quinone reductase has a crucial role in the oxidation of H_2S (REF. 10). The cytosol is the major intracellular site of H_2S methylation. Thiol *S*-methyltransferase catalyses the methylation of H_2S to yield methanethiol and dimethylsulfide.

H_2S is excreted in urine and flatus as free sulfate, thiosulfate or free sulfide, and is also exhaled in breath[11].

Tissue-specific distribution and intracellular compartmentalization of H_2S-generating enzymes. Among the mammalian tissues that express CSE are the cardiovascular system[12,13], liver, kidney, uterus, placenta, pancreatic islets[13,14], lung[15], and gastrointestinal tract[16–19]. Previously, although *CSE* mRNA had been detected in the brain, the lack of CSE expression in the nervous system was taken for granted[20]. However, a recent study revealed abundant expression of CSE proteins in the mouse and human striatum, cortex, and cerebellum[21].

The brain is the primary organ in which CBS expression is dominant. CBS protein has been identified in the hippocampus, cerebellum, cerebral cortex, and brainstem[22,23]. CBS expression and activities in other tissues, such as the liver, kidney, pancreas, gastrointestinal tract, and lungs, have been confirmed[5,6,12,17,19,24–26]. The expression of CBS protein in the cardiovascular system and the functional relevance of such expression have not been convincingly established. A recent study detected a CBS protein band in cardiac tissues[27], although such expression had not been detected in many previous studies. The answer to the controversy may not be simple, but certainly the specificity of the antibody used, the real identify of the protein band detected in western-blot analysis, and the use of appropriate positive and negative controls should be considered. These considerations and cautions also apply to the detection of CSE protein[28] and MST protein in western-blot analyses.

MST is expressed in the central nervous system, — mostly in glial cells[29], hippocampal pyramidal neurons, cerebellar Purkinje cells, and mitral cells in the olfactory bulb[30]. Both MST and CAT are detected in certain types of vascular endothelium. The expression of MST has also been detected in vascular smooth muscle cells[31], cardiomyocytes[29], kidney cells and liver cells[29,32].

Under physiological conditions, CSE proteins are mainly localized in the cytosol[33]. Increases in intracellular free calcium levels in vascular smooth muscle cells, due either to the entry of extracellular calcium or to the release of intracellular calcium from the endoplasmic reticulum, trigger the translocation of CSE from the cytosol to the mitochondrion[33]. It should be noted that the endogenous substance or substances that elicit CSE translocation to the mitochondrion have not yet been identified. It may also be possible that pathophysiological stimuli such as hypoxia directly increase intracellular calcium levels and result in the subsequent translocation of CSE. Interestingly, this mitochondrial translocation of CSE is not observed in hepatocytes[5].

CBS has also been conventionally regarded as being compartmentalized to the cytosol. Recent studies in liver cells[5] and colon-cancer-derived epithelial cells[26] show the existence of CBS proteins in the mitochondrion under resting conditions. MST and CAT are located both in the cytosol and in mitochondria[6,30].

Based on the tissue-specific expression patterns of various H_2S-generating enzymes, one may estimate the major enzymatic sources of H_2S in various organs and cells. H_2S production in the cardiovascular system is predominantly regulated by CSE, whereas CBS is the enzyme mostly responsible for H_2S production in the nervous system. In other systems and organs, multiple enzymes may control the production of H_2S, and their individual contributions may vary depending on the developmental stage and any disturbance of homeostasis. However, the above estimation clearly oversimplifies the complex nature of endogenous H_2S metabolism. For example, even within the central nervous system, different types of neurons or glial cells may rely on CSE or MST rather than CBS to produce H_2S. Furthermore, the endogenous production of H_2S depends on substrate availability and other properties of the intracellular milieu that may differentially affect enzyme activities, in addition to the expression and distribution of these enzymes.

Cellular and molecular effects of H_2S

H_2S participates in the regulation of homeostasis of numerous systems in our body, including but not limited to the cardiovascular, neuronal, gastrointestinal, respiratory, renal, liver and reproductive systems. The lipid-soluble nature of H_2S enables this gasotransmitter to easily reach its molecular targets — on the plasma membrane, inside the cytosol or in intracellular organelles. This ubiquitous membrane permeability underlies the wide scope of the physiological or biological effects of H_2S, but it is its unique chemical reactivity with certain types of macromolecules in different types of cells that makes H_2S a selective, specific and powerful signalling molecule.

Interactions with ion channels. Numerous cellular effects of H_2S are mediated by its interactions with membrane ion channels. The ATP-sensitive potassium (K_{ATP}) channel was the first-identified molecular target of endogenous H_2S, and it mediates H_2S-induced vasorelaxation[13]. The activation of K_{ATP} channels by H_2S has been reported in cardiovascular[13,34], endocrine[35], respiratory[36], nervous[37,38] and gastrointestinal systems[39,40]. It seems that multiple subunits of the K_{ATP} channel complex are modified by H_2S. Using a whole-cell patch-clamp technique and a mutagenesis approach, Jiang *et al.*[41] demonstrated that H_2S specifically acts on the sulfonylurea receptor 1 (SUR1; also known as ABCC8) subunit of the K_{ATP} channel complex to activate these channels. Specifically, the amino-acid targets

Cysteine aminotransferase (CAT). An enzyme that catalyses the conversion of L-cysteine and α-ketoglutarate to 3-mercaptopyruvate and glutamate. Another enzyme, 3-mercaptosulfurtransferase, can then catabolize the generation of hydrogen sulfide from 3-mercaptopyruvate.

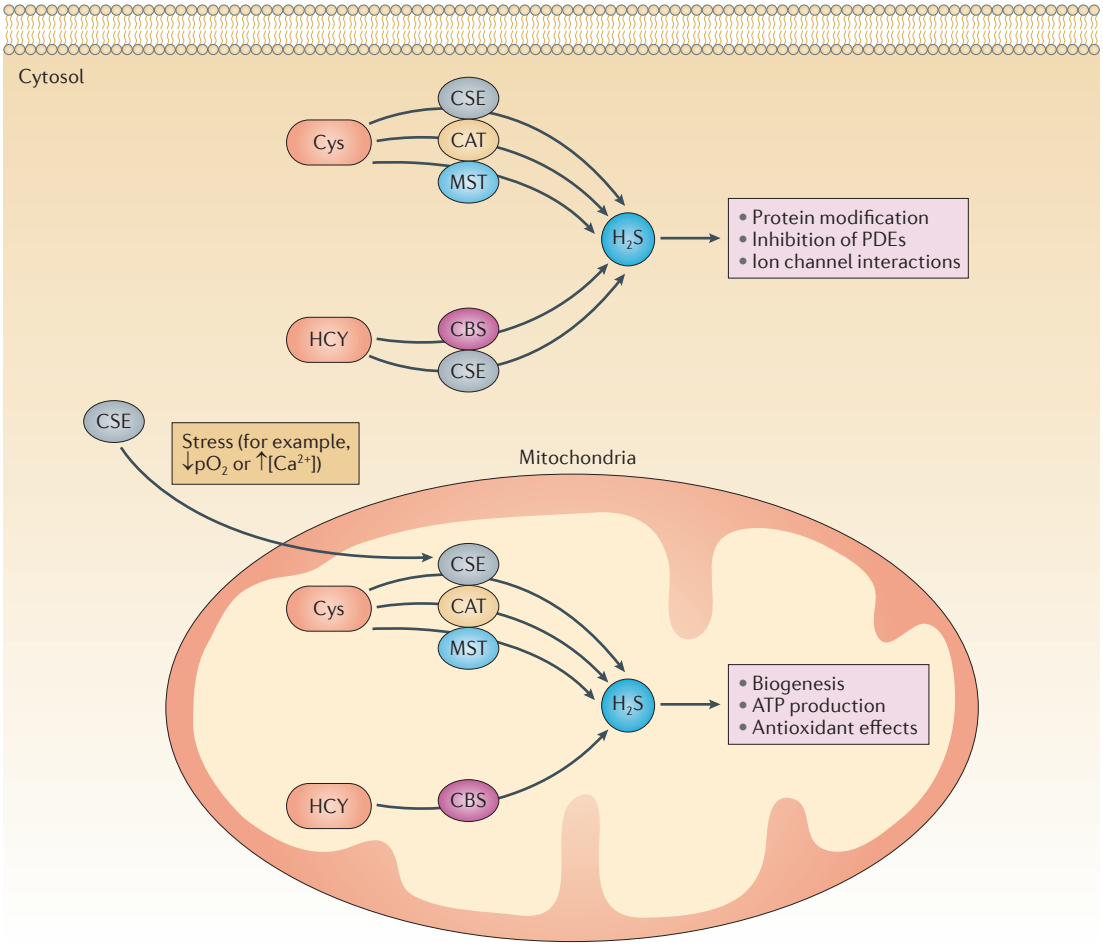


Figure 1 | **Cytosolic and mitochondrial production and functions of H_2S.** Depending on the cell type and specific stress conditions, endogenous hydrogen sulphide (H_2S) production can occur in the cytosol and/or the mitochondria[6]. Cystathionine γ-lyase (CSE)-mediated H_2S production usually occurs in the cytosol; however, under stress conditions CSE can be translocated from the cytosol into the mitochondrion, where it catalyses H_2S production from cysteine (Cys). Cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (MST) are located both in the cytosol and mitochondria, and regulate H_2S production. Cystathionine β-synthase (CBS) is located in the cytosol, but can also be found in the mitochondrion of certain cell types under resting conditions. Hypoxia decreases the degradation of CBS in the mitochondrion, leading to an accumulation of CBS and thus increasing production of H_2S in this organelle. HCY, homocysteine; PDE, phosphodiesterase; pO_2, partial pressure of oxygen.

of H_2S are the Cys6 and Cys26 residues of the extracellular N terminus of the SUR1 subunit. The SUR2B subunit of K_{ATP} channels in colonic circular smooth muscle cells is *S*-sulfhydrated by sodium hydrosulfide (NaHS); this action underlies the increased K_{ATP} channel current that is induced by NaHS and blocked by the sulfonylurea drug glybenclamide[42]. Similarly, the pore-forming subunit of the K_{ATP} channel (such as $K_{ir}6.1$) in vascular smooth muscle cells can be *S*-sulfhydrated, leading to increased K_{ATP} channel current amplitude and membrane hyperpolarization[34,43].

Voltage-dependent calcium channels (VDCCs) are also important targets of H_2S in various cell types. In isolated mouse pancreatic β-cells[44] and rat cardiomyocytes[45], H_2S inhibits L-type VDCC currents. The amplitude of the current through heterologously expressed recombinant voltage-gated calcium channel 3.2 ($Ca_v3.2$)-subunit T-type VDCCs in HEK293 cells was also inhibited by NaHS (at concentrations of 10 μM–1 mM)[46]. Indirect evidence also showed that native T-type VDCCs in rat cardiomyoblasts (H9c2 cells) were inhibited by H_2S, as Ni^{2+} (which inhibits these channels) abolished NaHS-induced decreases in resting intracellular calcium concentration[47]. However, the effects of H_2S on VDCCs appear to be tissue-specific; in neurons, the activities of $Ca_v3.2$ T-type VDCCs and $Ca_v1.2$ L-type VDCCs are enhanced by endogenous and/or exogenous H_2S[48,49].

An important advance in vascular physiology in recent years is the identification of H_2S as an endothelium-derived hyperpolarizing factor (EDHF)[48,49]. H_2S activates small- and intermediate-conductance calcium-activated potassium channels (SK_{Ca} channels and IK_{Ca} channels, respectively), and H_2S-induced endothelium-dependent smooth muscle hyperpolarization and vasorelaxation of mouse mesenteric arteries are abolished by the co-application of charybdotoxin and

Box 1 | **Determination of endogenous H_2S levels**

Determining endogenous hydrogen sulfide (H_2S) levels in the circulation and in tissues and cells is important for characterizing H_2S as a gasotransmitter. It is also needed for evaluating whether exogenous H_2S donors mimic the physiological and biological effects of endogenous H_2S. As has been systematically reviewed recently[176], a wide range — from 0.1 μM to more than 300 μM — of circulating H_2S levels has been reported under physiological conditions. This remarkable variation is largely attributable to the wide array of measurement methods that have been used, including the colorimetric methylene blue method, ion-selective or polarographic electrodes, gas chromatography with flame photometry and the monobromobimane assay. Often, the total reactive sulfur pool — which does not distinguish free H_2S from acid-labile sulfide or bound sulfur (also known as sulfane-sulfur) — was measured. The extent of H_2S oxidation and/or H_2S scavenging in biological samples may also skew estimations of the true concentrations of H_2S. For example, it was reported that plasma levels of H_2S could not be reliably measured, as red blood cells avidly scavenge H_2S and remove it from the circulation[177].

These measurement pitfalls have been addressed through the use of more advanced techniques. By measuring the H_2S in headspace gas from plasma samples, Shen *et al.*[178] showed that the human plasma sulfide level is around 3 μM. Using the monobromobimane method coupled with reverse-phase high-performance liquid chromatography, the same authors recorded free hydrogen sulfide levels of 0.2–0.8 μM and acid-labile sulfur levels of 1.8–3.8 μM in plasma from mice and from humans[178]. With a combined modified gas chromatography and mass spectrometry technique, H_2S levels of 0.5–2.5 μM were measured in pig and mouse blood[176]. Thus, as a conservative estimate, plasma H_2S levels in healthy humans or animals are in the higher nanomolar to lower micromolar range[178–180]. However, there is still no consensus on the physiological levels of H_2S in blood, or on the most acceptable and reliable techniques for measuring H_2S in biological samples.

With respect to determining the physiological importance of H_2S, does it matter if the circulatory or tissue levels of endogenous H_2S are in the nanomolar or micromolar range? It seems not. At nanomolar or even picomolar concentrations, numerous endogenous substances exert profound physiological effects in different systems. Indeed, in many cases, it is the relative change in endogenous H_2S levels that is more important in determining the physiological and pathophysiological importance of this gasotransmitter. This notion is exemplified by reports of the development of hypertension[94], atherosclerosis[101], diabetes[119], renal ischaemic damage[180], colitis[155], acute liver failure[153,181] and Huntington disease[21] owing to reduced gene expression or activity of cystathionine γ-lyase (CSE).

As far as the issue of free H_2S versus total reactive sulfur is concerned, what is important is the indication and interpretation of the measured levels, not the methods themselves. Ideally, we should precisely and reliably detect changes in each and all components of the total sulfur pool. Given that free H_2S, acid-labile sulfide, and sulfane-sulfur are interchangeable under different conditions, an understanding of the mechanisms underlying the changes in this total reactive sulfur pool, and the physiological effects of such changes, carry biological and therapeutic importance.

However, should variations in H_2S levels be used as biomarkers for the purpose of diagnosis and prognosis of certain diseases, it becomes imperative to accurately measure the absolute concentrations of H_2S in the circulation and in tissues. Furthermore, the development of H_2S-based therapies will be facilitated by accurately monitoring H_2S levels in the circulation and/or in the targeted organs and systems in order to determine the pharmacokinetics and pharmacodynamics of H_2S donors as well as their efficacy and toxicity profiles.

apamin[50]. The co-application of these two compounds specifically blocks IK_{Ca} channels and SK_{Ca} channels, and, as such, should block the vasorelaxant effect of an EDHF. The molecular basis for the role of H_2S as an EDHF is the post-translational modification of the targets of H_2S. H_2S can sulfhydrate IK_{Ca} channels in primary human aortic endothelial cells[51]. Furthermore, in vascular tissues the expression of $SK_{Ca}2.3$ channels but not $IK_{Ca}3.1$ channels was increased by H_2S and decreased by a CSE inhibitor or by deletion of the gene encoding CSE[50].

The inhibitory effects of H_2S on the α-subunit of big-conductance calcium-activated potassium (BK_{Ca}) channels in heterologously transfected HEK293 cells have been reported[52]. Likewise, H_2S inhibited native BK_{Ca} channels in type 1 glomus cells from the isolated mouse carotid body[53]. By sharp contrast, ~300 μM NaHS (an H_2S donor) increased whole-cell BK_{Ca} currents and enhanced single-channel BK_{Ca} activity in rat pituitary tumour cells[54]. This stimulatory effect of NaHS (10 μM) on BK_{Ca} channels was also observed in endothelial cells[55]. These differential effects may be attributable to specific subtypes of BK_{Ca} channels in different types of cells and to the concentrations of H_2S donors.

The interaction of H_2S with ion channels is not restricted to K_{ATP} and K_{Ca} channels. Delayed-rectifier K^+ channels in mouse gastric-muscle cells can be inhibited by H_2S (REF. 56), whereas 4-aminopyridine (4-AP)-sensitive K^+ channels in rat coronary artery smooth muscle cells can be activated by H_2S[57]. H_2S has also been reported to activate chloride channels, voltage-gated sodium $Na_v1.5$ channels and the transient receptor potential cation channels TRPV1 and TRPA1 in different tissues[58].

As the physiological level of endogenous H_2S in the circulation or in various types of cells is unclear, many of the aforementioned studies, such as those describing the effects of H_2S on BK_{Ca} channels, cannot be used as evidence for a physiological role of H_2S in the regulation of different ion channels. Indeed, establishing such a physiological role of H_2S would require a comparison of the characteristics of ion channels in the presence and absence of endogenous H_2S. This forethought should be taken into account in all other studies that use H_2S donors to deduce the physiological actions of H_2S. Moreover, ion channels are diversified in their amino-acid composition, voltage dependency, gating mechanisms,

4-aminopyridine
(4-AP). One of three isomeric amines of pyridine, which is widely used as a research tool to characterize the subtypes of potassium channels.

ion selectivity and sensitivity to different endogenous signalling molecules. This diversity forms the basis of the variable responses of ion channels to H_2S exposure.

Interactions with classical second messengers. Although H_2S can directly act on its target proteins without the engagement of second messengers, this gasotransmitter also affects the levels of several known second messengers. Non-selective inhibition of phosphodiesterases (PDEs) by H_2S leads to decreased degradation of cyclic GMP and cyclic AMP, thereby increasing net cGMP and cAMP levels[59–61]. For example, with regard to cAMP levels, it has been demonstrated that H_2S inhibits the mitochondrial-matrix-localized PDE2A in isolated rat liver mitochondria — leading to increased cAMP levels — as well as inhibiting the recombinant PDE2A enzyme *in vitro*[51]. Conversely, H_2S can inhibit adenylyl cyclase in the rat kidney[62]. Thus, the net effect of H_2S on cAMP levels could be determined by assessing the balance between the inhibition of PDEs and the inhibition of adenylyl cyclase.

Intracellular free calcium is another second messenger for cellular signal transduction. Via its effects on plasma membrane ion channels and on intracellular calcium pools, H_2S can increase intracellular calcium levels in vascular endothelial cells by stimulating calcium influx[63] or by releasing calcium from an ATP- and 4-chloro-3-ethylphenol (4-CEP)-sensitive intracellular pool[64]. The consequent elevation of intracellular calcium levels may activate many calcium-dependent signalling pathways and enzymes such that endothelial proliferation and function can be regulated. H_2S decreases intracellular calcium levels in cardiomyocytes by inhibiting both L-type and T-type VDCCs, but it increases intracellular calcium release in the same cell preparation[47]. The functional consequence of this biphasic effect of H_2S is not clear. H_2S increases calcium sparks in smooth muscle cells of intact piglet cerebral arterioles[65] and mesenteric arteries[55]. Increased calcium sparks activate transient K_{Ca} channels and smooth muscle hyperpolarization; eventually, global intracellular free calcium levels drop and vasodilation ensues[65].

Protein* S-*sulfhydration. At the amino-acid level, NO and H_2S both modify sulfhydryl groups of certain proteins, but often generate opposite effects[66]. NO covalently modifies free sulfhydryls (–SH) of the cysteine residues of these targeted proteins to form *S*-nitrosothiols. Such *S*-nitrosylated proteins usually have decreased functions. Low-molecular-weight thiols or cysteine residues of proteins can also be modified via *S*-sulfhydration, in which the –SH from a sulfhydryl donor is transferred to cysteine sulfhydryls, forming a covalent persulfide (–SSH) in the target protein[51].

The mechanisms for H_2S-induced protein *S*-sulfhydration are under debate. As the sulfur in H_2S and in –SH groups is at its lowest oxidative state, H_2S may not be able to directly interact with the –SH group of cysteine to form a persulfide, and the oxidation of cysteine and/or the production of polysulfides may be prerequisite for protein *S*-sulfhydration.

The abundance of *S*-sulfhydrated proteins *in vivo* has also been questioned. Whereas a previous study using the biotin-switch assay reported that 10–25% of liver proteins are *S*-sulfhydrated[43], another study using a tag-switch method revealed a much lower abundance of protein *S*-sulfhydration[67]. The pH of the cellular environment, and the proximity of the target cysteine amino acids to the active centrum of the protein would affect the extent of protein *S*-sulfhydration. Therefore, special attention should be paid to the sample preparation, including the oxidative state and level of protonation of cysteine thiol groups, in order to more accurately determine the extent of protein *S*-sulfhydration.

S-sulfhydration can affect numerous proteins[68]. For example, Kelch-like ECH-associated protein 1 (KEAP1) is a negative regulator of the activity of nuclear factor erythroid 2-related factor 2 (NRF2). H_2S-induced *S*-sulfhydration of KEAP1 at Cys151 facilitates the dissociation of KEAP1 from NRF2, thereby resulting in an enhancement of NRF2-mediated antioxidant responses[14].

The post-translational modification of nuclear factor-κB (NF-κB) serves as an example of the difference in functional effects between *S*-nitrosylation and *S*-sulfhydration. NF-κB is a transcription factor that regulates the expression of many inflammation- and apoptosis-responsive genes. *S*-nitrosylation of the Cys38 residue of the p65 subunit of NF-κB inhibits the binding of NF-κB to the cytokine-responsive sites in the promoter of the gene that encodes inducible NO synthase, resulting in decreased expression of this enzyme[69]. *S*-sulfhydration of the same cysteine residue, however, enhances the binding of NF-κB to ribosomal protein S3, which increases the transcriptional activity of p65 in the nucleus. Consequently, cell apoptosis is greatly inhibited[68].

For a signal transduction process to be regulated, both 'turn-on' and 'turn-off' mechanisms are required. For example, the removal of NO from *S*-nitrosylated proteins occurs through the de-nitrosylation process. S-nitrosoglutathione reductase (GSNOR), a de-nitrosylation enzyme, reduces the product of protein *S*-nitrosylation, GSNO, to glutathione hydroxysulfenamide[70]. Thioredoxin, another de-nitrosylation enzyme[71], has two redox-active cysteine residues (in the sequence Cys-Gly-Pro-Cys) in its active site. Thioredoxin breaks the disulfide bonds of its target protein and then binds with the same protein; this activity accounts for its antioxidant function. By de-nitrosylating its substrates, thioredoxin can reverse the function of *S*-nitrosylated proteins. However, our knowledge of protein de-sulfhydration is very limited. Mitochondrial persulfide dioxygenase (ETHE1) has been shown to catalyse the conversion of glutathione persulfide (GSSH) to sulfite *in vitro*. This process has important implications for mitochondrial sulfur catabolism and redox balance[72]. However, the physiological substrate for ETHE1 is not known, although the persulfide bound in the active-site peptide of sulfide-quinone oxidoreductase might be one candidate. Moreover, little is known about the de-sulfhydration of *S*-sulfhydrated proteins in the cytosol or non-mitochondrial organelles. It is conceivable, however, that the *S*-sulfhydrated proteins may be de-sulfhydrated

Calcium sparks
Intracellular Ca^{2+} release events that play an important role in excitation–contraction coupling.

Tag-switch method
A technique used to measure protein *S*-sulfhydration, whereby *S*-sulfhydrated residues are labelled to form thioether conjugates.

in the presence of excess reductants and, as such, the level of oxygen will be a factor in the de-sulfhydration process.

Role in redox balance. Redox is the integration of reduction and oxidation processes, in which electrons are transferred between species. Redox balance reflects a dynamic state in which oxidant and antioxidant levels are balanced. Increased oxidative stress and/or decreased antioxidant capability under various pathological situations disrupt the redox balance and cause damage to molecules and cells. The protective, antioxidant actions of H_2S have been well established, particularly with respect to the vasculature. In cultured vascular smooth muscle cells, oxidative stress that was induced by high levels of homocysteine or methylglyoxal was markedly reduced by NaHS at concentrations of 30–90 μM (REFS 73,74). Moreover, the accumulation of lipid peroxidation products (including lipid hydroperoxide, the main contributor to the cytotoxic effect both of oxidized low-density lipoprotein (LDL) and of lipid derived from atheroma) in human umbilical vein endothelial cells (HUVECs) was inhibited or prevented by NaHS at 1–20 μM; endothelial cytotoxicity mediated by hydrogen peroxide and oxidized LDL was similarly inhibited or prevented by NaHS at these concentrations[75]. Oxidative stress-mediated death of amyloid-β-treated microglial cells was also inhibited by NaHS at concentrations as low as 25 μM (REF. 76). Although the physiological relevance of the antioxidant effect of H_2S at these concentrations is unclear, the therapeutic value of H_2S donors in this regard is certainly apparent.

In addition to its reducing nature, H_2S influences the oxidant–antioxidant balance. Expression of the antioxidant enzyme thioredoxin 1 in vascular endothelial cells is upregulated by H_2S (REF. 77), whereas the expression of NAD(P)H oxidase, a key source of extramitochondrial oxidant species, is downregulated in osteoblastic cells that have been exposed to H_2S (REF. 78). The production of glutathione (GSH), a strong scavenger of free radicals, is also enhanced by H_2S. It was shown that incubation of cultured mouse embryonic fibroblasts with NaHS induces nuclear translocation of NRF2 by *S*-sulfhydrating KEAP1 at Cys151. As such, there was an increase in the binding of NRF2 to the antioxidant-response element (ARE), and an increase in ARE-mediated transcription of the genes involved in GSH synthesis and maintenance. The consequent increase in GSH levels contributes to the protective effect of H_2S against cellular senescence induced by oxidative stress[14].

The mitochondrion is the primary source of oxygen-derived free radicals and the organelle that is most affected by oxidative stress. Oxidative stress suppresses mitochondrial electron transport and bioenergetics, but these effects can be reversed by exogenous H_2S (REF. 79). It was recently reported that nanomolar concentrations of a novel mitochondrion-targeted H_2S donor, AP39, inhibited oxidative damage in microvascular endothelial cells *in vitro*[80]. GYY4137, a non-mitochondrion-targeted, slow-releasing H_2S donor, produced protective effects similar to those produced by AP39, but only at a 1,000-fold higher concentration than that of AP39 (REF. 81).

Oxygen sensing and mitochondrial bioenergetics. Changes in the oxygenation status of cells affect endogenous H_2S levels in different ways. Under hypoxic conditions, the oxidation of H_2S in mitochondria is decreased, leading to a net elevation of H_2S levels. In vascular smooth muscle cells, hypoxia or calcium overloading also leads to the translocation of CSE from the cytosol to mitochondria, where CSE uses approximately threefold higher concentrations of L-cysteine to produce H_2S (REF. 33). In addition, whereas under normoxic conditions CBS in hepatocytes is degraded inside mitochondria by Lon protease[5], upon hypoxic stress Lon protease cannot recognize the deoxygenated haem group in CBS, and therefore CBS is not degraded. The accumulation of CBS protein in the mitochondrion leads to increased mitochondrial production of H_2S (REF. 5).

It appears that different tissues handle changes in the partial pressure of oxygen differently by altering their H_2S production and oxidation, as well as by changing their responses to H_2S. For example, H_2S dilates systemic blood vessels[82] and/or promotes angiogenesis to increase blood supply to hypoxic tissues[83]. By contrast, in hypoxic rat lungs, H_2S constricts vascular smooth muscle in order to achieve regional ventilation–perfusion matching, in a mechanism known as hypoxic pulmonary vasoconstriction[84,85]. In this way, H_2S helps the diversion of blood from oxygen-deprived areas to oxygen-supplied areas[86]. At the same time, H_2S dilates airway smooth muscle to increase lung ventilation (in humans)[36] and to reduce airway resistance (in mice)[15]. These compensatory changes induced by H_2S in response to hypoxia help improve the efficiency of pulmonary gas exchange. Moreover, H_2S mediates the response of carotid-body chemoreceptors to hypoxia by modulating BK_{Ca} channels[53]. As far as bioenergy production is concerned, under hypoxic conditions the upregulated mitochondrial production of H_2S — which can act as an electron donor in the mitochondrial respiratory chain — contributes to the generation of bioenergy[79]. Thus, in facing a hypoxic challenge, mitochondrial production of ATP can be maintained by hypoxia-increased H_2S levels[33,86,87].

It should be mentioned that the notion of an 'oxygen sensor' role of H_2S does not go without challenge[88]. A decrease in the partial pressure of oxygen in pulmonary circulation does not always lead to an increase in H_2S levels, and H_2S does not always cause the constriction of pulmonary arteries. For instance, in hypoxic pulmonary hypertension, hypoxia and a reduction of H_2S levels in the pulmonary circulation occur in parallel[89]. In another example, in contrast to the assumed vasoconstrictive effect of H_2S on pulmonary arteries, one recent study showed that, under normoxic conditions, H_2S actually dilated pre-constricted human lobar pulmonary artery rings and reduced pulmonary artery pressure[90]. It is not yet clear whether the vasoactive effects of H_2S on pulmonary circulation, and the interaction of H_2S with hypoxia, are species-specific.

H_2S in disease processes

H_2S levels contribute to homeostasis of the organism, and abnormally increased or decreased endogenous H_2S production is associated with various diseases. Among the most-studied diseases related to abnormal

H_2S metabolism are those involving the cardiovascular, endocrine, gastrointestinal and nervous systems. For the pathological roles of H_2S in other mammalian systems, readers are referred to other recent review articles[2,5,91].

Hypertension and vascular remodelling. As an EDHF, H_2S induces the relaxation of many systemic blood vessels. When it is produced in smooth muscle cells or other non-endothelial cells, H_2S also dilates blood vessels by directly relaxing smooth muscle cells[12,92]. These endothelium-dependent and -independent vasorelaxant effects of H_2S are linked to the role of H_2S in blood pressure regulation. Since the early study by Zhao *et al.*[93] in rats that showed that pharmacological inhibition of CSE elevated blood pressure, the role of H_2S in regulating blood pressure has become increasingly clear. Mice that are genetically deficient in CSE (CSE-knockout mice) exhibit an age-dependent development of hypertension that is largely due to diminished endothelial production of H_2S and endothelium-dependent relaxation of peripheral resistance arteries[82]. Similarly to CSE-knockout mice, spontaneously hypertensive rats (SHRs) exhibit age-dependent development of hypertension that correlates with diminished CSE expression and H_2S production in aortic tissues[82,94]. The treatment of CSE-knockout mice with NaHS at 3.9 μmol per kg, 19.5 μmol per kg, and 39 μmol per kg (intravenous administration) or of SHRs with NaHS at 56 μmol per kg (intraperitoneal (i.p.) administration) suppressed the development of hypertension, decreased vascular damage and prevented vascular remodelling[82,94,95].

Altered H_2S metabolism has been linked to pulmonary hypertension. After high pulmonary blood flow-induced pulmonary hypertension was established in rats after an abdominal aorta–inferior cava vein shunt operation, the plasma level of H_2S and *CSE* mRNA levels in lung tissues were significantly lower than in normotensive control rats[96]. In another rat model of hypoxia-induced pulmonary hypertension, the animals were exposed to normobaric hypoxia (10% oxygen) in a transparent plastic hypoxia chamber for 3 weeks. In these hypertensive rats, H_2S production and CSE expression in lung tissues decreased significantly compared with that in control rats[89,97]. In rats with hypoxic pulmonary hypertension, daily treatment with NaHS at 14 μmol per kg (i.p. administration) provided anti-hypertensive protection in these animals and reduced the extent of remodelling of pulmonary arteries[89,97].

In the two-kidney-one-clip model of renovascular hypertension, upregulated renin expression and increased activity of plasma renin and angiotensin II are markers of the severity of the disorder. As reflected by these measures, systemic hypertension was either prevented or attenuated by daily administration of NaHS (at doses of 30–100 μmol per kg) to rats in this model[62].

The role of H_2S in gestational hypertension has also been investigated. Hypertension is one of the major characteristics of pre-eclampsia — a disorder that affects women during pregnancy, but that has no clear aetiology. Endogenous H_2S is required for healthy placental vasculature, whereas decreased CSE or H_2S activity may contribute to the pathogenesis of pre-eclampsia. In pregnant mice, the inhibition of CSE with propargylglycine induced hypertension and promoted abnormal labyrinthine vascularization in the placenta[98]. Women with pre-eclampsia exhibit decreased plasma levels of H_2S and reduced expression of CSE in the placenta compared with gestational, age-matched controls[98], and the therapeutic value of H_2S donors (NaHS or GYY4137) in pre-eclampsia has been shown in mice[98] and rats[99].

Angiogenesis and atherosclerosis. H_2S increases vascular endothelial cell proliferation and migration, microvessel formation and the healing of wounds and ulcers both *in vivo* and *in vitro*. These pro-angiogenic effects of H_2S are proposed to be mediated through the phosphorylation of AKT, extracellular signal-regulated kinase (ERK) and p38, as well as through the activation of K_{ATP} channels[18,83,92,100]. The interaction of vascular endothelial growth factor (VEGF) and H_2S constitutes another important pro-angiogenesis mechanism. Rats treated with NaHS (50 μmol per kg, twice daily) showed increased free plasma levels of VEGF and upregulated renal expression of *Vegfa* mRNA. *In vitro* incubation of podocytes with NaHS resulted in VEGF release and upregulation of *Vegfa* mRNA levels[99]. In turn, VEGF stimulates the release of H_2S from vascular endothelial cells[83]. Thus, H_2S and VEGF individually and synergistically stimulate angiogenesis. Similarly, the interaction between H_2S and NO can affect angiogenesis[60,66,100]. NaHS was shown to promote the phosphorylation of endothelial NO synthase in cultured HUVECs, leading to increased production of NO, which further contributed to endothelial cell proliferation and tube formation[100].

Atherosclerosis is a chronic circulatory disease that is characterized by the build-up of fatty or high-cholesterol plaques on the inner surface of large- to medium-sized blood vessels. The pathogenesis of atherosclerosis is not fully understood, but recent studies have indicated that altered H_2S metabolism is involved in both the initiation and progression of this vascular disorder. Atherosclerosis in apolipoprotein E (APOE)-knockout mice is accompanied by decreased H_2S levels in the blood, and treatment of these mice with NaHS was shown to attenuate the thickening and stiffening of arterial vessels[101]. Mani *et al.*[102] showed that CSE-knockout mice that were fed an atherogenic paigen-type diet for 12 weeks developed early fatty-streak lesions in the aortic root, increased oxidative stress and expression of adhesion molecules, and enhanced aortic intimal proliferation. By contrast, wide-type mice fed the same atherogenic diet, or CSE-knockout mice fed a normal diet, did not develop any atherosclerotic damage. The anti-atherosclerotic effects of H_2S are manifested through: the inhibition of neointimal hyperplasia and smooth muscle proliferation; a decrease in levels of oxidized LDLs, vascular calcification and vascular inflammation; and a suppression of the adhesion of monocytes to endothelial cells[102,103].

An enhanced understanding of the anti-atherosclerotic role of H_2S has helped advance H_2S-based therapies targeting atherosclerosis. In APOE-knockout mice, treatment with NaHS (56 μmol per kg per day, i.p. administration) or GYY4137 (133 μmol per kg per day, i.p.

Two-kidney-one-clip model
A model of hypertension induced by chronically constricting one renal artery while the other renal artery remains fully perfused.

administration) reduced the atherosclerotic plaque load and partially restored acetylcholine-induced endothelium-dependent relaxation of the aorta[101,103]. H_2S donors also suppressed the expression of intercellular adhesion molecule 1 (ICAM1), tumour necrosis factor (TNF) and interleukin-6 (IL-6) in the aorta; the expression of CX3C-chemokine receptor 1 (CX3CR1) and CX3C-chemokine ligand 1 (CX3CL1) in macrophages and lesion plaques; and the generation of superoxides in the aorta of these animals[101,103,104]. Moreover, in these mice, early treatment with NaHS at a dose of 1 mg per kg per day (i.p. administration) generated better therapeutic benefit against atherosclerotic damage than did NaHS after atherosclerosis had fully developed[104].

Lipid metabolism disorders and liver diseases. The liver has essential roles in lipid metabolism, and hepatic production of H_2S plays a key part in managing lipid metabolism. Indeed, genetic deficiencies in either CBS or CSE result in hyperhomocysteinaemia. CBS-knockout mice that are fed normal chow exhibit abnormal lipid metabolism, with increased serum and hepatic levels of triglycerides and non-essential fatty acids, as well as spontaneous hepatic fibrosis and steatosis[105,106]. Nevertheless, liver morphology and function seem to be normal in chow-fed CSE-knockout mice[107]. The impact of CSE deficiency on lipid metabolism becomes detectable only after these mice are fed a high-fat atherogenic diet: with such an atherogenic feeding regime, they exhibit increased plasma levels of total and LDL cholesterol, decreased high-density lipoprotein (HDL) cholesterol and early development of atherosclerosis[102]. Strikingly, in humans, plasma H_2S levels are positively correlated with HDL cholesterol, and negatively correlated with the ratio of LDL to HDL cholesterol[108].

Downregulation of CSE expression and decreased endogenous H_2S levels in the liver are common consequences observed in bile duct-ligated rats with cirrhosis and associated portal hypertension[109] or in rats with carbon tetrachloride-induced liver cirrhosis[110]. Several studies have shown the beneficial effects of H_2S supplementation therapy in these models of liver diseases[109–111]. However, there are circumstances of elevated H_2S production in which treatment with H_2S donors can be detrimental. For example, when mice with septic shock were treated with NaHS at a dose of 14 μmol per kg (i.p. administration) this increased liver damage[112], whereas pharmacological inhibition of CSE with DL-propargylglycine (50 mg per kg, i.p. administration) protected these animals from liver damage. In rat endotoxaemia, portal infusion of sodium sulfide (Na_2S) through a splenic cannula at a rate of 2 μmol per kg per minute for 10 minutes caused hepatic vasoconstriction and increased portal pressure *in vivo*[113]. Conversely, treatment of the rats with DL-propargylglycine (50 mg per kg, i.p. administration) significantly attenuated the vasoconstrictive effect of endothelin 1 in endotoxin-treated animals. The proposed underlying mechanism for the detrimental effects of H_2S in these situations is that the perturbed hepatic sinusoidal perfusion during sepsis is further decreased by H_2S supplementation, thus leading to exacerbated tissue hypoxia[113].

Diabetes. Diabetes mellitus is caused by decreased bioavailability of insulin from the pancreas and/or diminished insulin sensitivity of the peripheral tissues. Altered metabolism of H_2S in the pancreas and in peripheral tissues is involved in both the pathogenesis of diabetes and its complications (FIG. 2). Overexpression of CSE and the consequential overproduction of H_2S in pancreatic β-cells, as detected in genetic diabetic rat models (Zucker diabetic rats), constitute pathogenic factors for diabetes[114]. Loss of functional β-cell mass is directly responsible for the pathogenesis of type 1 diabetes and progression of type 2 diabetes[115]. Although a β-cell-killing effect of H_2S has not been directly demonstrated *in vivo*, cultured insulin-secreting INS-1E cells undergo increased apoptosis in response to H_2S — a finding that is certainly indicative of the potential impact of H_2S on β-cell mass[116].

Also relevant to pancreatic insulin release is the H_2S-induced stimulation of K_{ATP} channels in β-cells, and their subsequent membrane hyperpolarization[35,114]. In addition, H_2S has been shown to inhibit L-type VDCCs in mouse pancreatic β-cells[44]. H_2S-induced membrane hyperpolarization and inhibition of VDCCs synergistically decrease calcium entry and insulin release from β-cells[44]. Following the consideration of the negative effects of H_2S on pancreatic insulin production and insulin release, a causative role of pancreatic H_2S in diabetes was proposed in 2004 (REFS 114,117). Zucker diabetic fatty rats have a type 2 diabetic phenotype. Pancreatic β-cell expression of the gene encoding CSE and β-cell production of H_2S were both found to be significantly higher in Zucker diabetic fatty rats than in non-diabetic Zucker lean or non-diabetic Zucker fatty rats[114,117]. Patients with type 1 or type 2 diabetes (but without nephropathy) had significantly enhanced activities of CSE and CBS compared with healthy controls[118,119]. Further study of Zucker diabetic fatty rats has shown that inhibition of CSE activity significantly decreased the production of H_2S, increased plasma insulin levels, and lowered hyperglycaemia[114]. Diabetes induced by streptozotocin was much slower to develop in CSE-knockout mice than in wild-type mice[120]. Moreover, streptozotocin induced a higher amount of pancreatic H_2S production and killed more β-cells in wild-type mice than in CSE-knockout mice[120]. Therefore, at least some of the diabetes-inducing effects of streptozotocin are mediated by CSE and/or H_2S, and an increased level of pancreatic H_2S is a causative factor for diabetes development.

What happens in the pancreas in terms of the metabolism and functions of H_2S is quite different to what happens in other parts of the body. Once diabetes is established, various diabetic complications can develop, including cardiomyopathy, nephropathy and vascular disorders associated with endothelial dysfunction. These complications are closely related to systemic H_2S deficiency, as hyperglycaemic cells oxidize and consume more H_2S (REFS 121,122). Overexpression of CSE or treatment with H_2S prevented dysfunction of cultured microvascular endothelial cells that was induced by high levels of glucose; improved hyperglycaemia-impaired endothelium-dependent aortic vascular relaxations *in vitro* and *ex vivo*[123]; and accelerated wound healing by restoring the functions of endothelial progenitor

Hyperhomocysteinaemia
A condition characterized by abnormally high levels of homocysteine in the blood. The major causes of this condition are deficiencies of vitamins B6, B9 and B12, and mutations in the gene encoding the enzyme 5-methyltetrahydrofolate.

Streptozotocin
A chemical commonly used to induce diabetes in laboratory animals, as it is toxic to the insulin-producing β-cells in the pancreas.

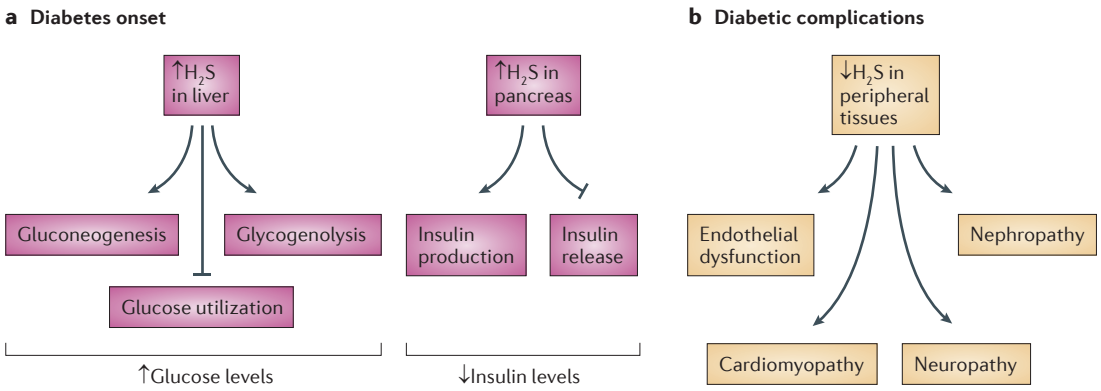


Figure 2 | **The pathogenic roles of H_2S at different stages of diabetes development.** Elevated endogenous hydrogen sulfide (H_2S) levels in pancreatic β-cells and hepatocytes have crucial roles in the onset of diabetes. At late stages of the disease, endogenous H_2S levels are lower in the affected organs and tissues, such as in vascular endothelial cells or cardiomyocytes, contributing to the development of diabetic complications in these organs. Correspondingly, H_2S-based therapy for diabetes should be staged in two phases. At the onset phase of diabetes (part **a**), selective inhibition of the endogenous production of H_2S in pancreatic β-cells and the liver would both increase insulin availability and decrease glucose production. By contrast, at later stages (part **b**), selective delivery of H_2S donors to the peripheral organs would be beneficial for preventing and treating diabetic complications.

cells and the activation of angiopoietin 1 signalling[124]. Notably, in rats with streptozotocin-induced diabetes, NaHS treatment at 100 μmol per kg per day (i.p. administration) also improved cardiac function and reversed the diabetes-related cardiac morphological changes by protecting cardiomyocytes against oxidative damage and preserving mitochondrial functions[125].

Neurodegenerative diseases. Brain levels of H_2S in individuals with Alzheimer disease are lower than in age-matched healthy people, although the expression levels of CBS between these groups are not different[126]. As Alzheimer disease is associated with reduced production of H_2S, there may be an associated decrease in neuronal cytoprotection, such that the deleterious effects of damage and neuroinflammation induced by amyloid-β and oxidative stress are increased[127–129]. Whether the low levels of H_2S in the brain observed in Alzheimer disease are a cause or a consequence of the disorder is not clear. In a rat model of ischaemic vascular dementia, plasma H_2S levels were lower and inversely correlated with the decrease in the number of viable neurons in the hippocampus. Intraperitoneal injection with NaHS (14 μmol per kg) markedly protected against neuronal injury and improved the performance of learning and memory, tested by the Morris water maze, in these animals[130].

Parkinson disease is another neurodegenerative disease in which the metabolism of H_2S may be involved. In a mouse model of Parkinson disease, H_2S levels in the substantia nigra and striatum were significantly lower than in control mice[131]. H_2S that was given via injection[131] or inhalation[132] impeded or prevented Parkinson-disease-like abnormalities, including movement dysfunction and microglial activation.

Unexpectedly — given the dominant expression of CBS in the brain — a recent study revealed the importance of CSE for the manifestation of Huntington disease (HD), an autosomal-dominant disease associated with a mutation in the gene encoding huntingtin[21]. In this study, CSE deficiency was found in brain tissues (the striatum and cerebral cortex) but not in the cerebellum of patients with HD, keeping in line with the relative susceptibility of these brain regions to HD damage. Furthermore, in murine models of HD (Q175 mice and R6/2 HD mice), CSE expression was downregulated in the striatum, cortex, hippocampus, hypothalamus and brainstem, but not in the cerebellum. Interestingly, CSE-knockout mice display impaired rotarod performance and an abnormal hindlimb clasping and clenching phenotype that is reminiscent of murine models of HD. These HD-related phenotypic changes were reversed by exogenously supplied cysteine[21].

In traumatic spinal cord injury, neuronal damage is initially caused by the trauma itself, but considerable additional damage is caused by the ensuing inflammatory reaction. In a mouse model of spinal cord injury, Campolo *et al.*[133] demonstrated the potential of using H_2S to decrease the inflammatory component of the injury, and to accelerate the recovery of lost motor function. Post-trauma treatment with a nonsteroidal anti-inflammatory drug (NSAID), naproxen, enhanced recovery of lost motor function in the mice, and decreased several indices of spinal cord inflammation. However, in mice that were treated with an H_2S-releasing derivative of naproxen (ATB-346), there was a marked acceleration in the recovery of lost motor function and further enhancement of anti-inflammatory effects.

Gastrointestinal disorders. H_2S, including that produced by enteric bacteria (BOX 2), has been implicated as a mediator of several physiological functions in the digestive tract. There is emerging evidence of the utility of H_2S donors in treating several gastrointestinal disorders, particularly those associated with inflammation (FIG. 3).

For example, abdominal pain is a common but poorly treated condition, and chronic abdominal pain is often described as irritable bowel syndrome.

The role of H_2S in modulating visceral pain is controversial: conflicting data suggest that it is both pro- and anti-nociceptive[39,91,134–137]. However, these differences may be related to the models used and the types and doses of H_2S donors that were used[91]. Several studies have suggested potent anti-nociceptive effects of H_2S donors in rodent models of visceral pain[39,91,134,136]. For example, colonic distention-induced pain in rats was substantially attenuated by several H_2S donors, the actions of which were mediated, at least in part, through the activation of K_{ATP} channels[39]. Similarly, gastric distention-induced pain in rats was markedly reduced by H_2S donors and exacerbated by an inhibitor of CSE activity[91]. An H_2S-releasing salt of trimebutine (an opioid anti-spasmotic drug) was shown to be safe and well tolerated in a Phase I clinical trial (ClinicalTrials.gov identifier: NCT01738425)[138], and is now being tested as an abdominal analgesic in Phase II clinical trials (ClinicalTrials.gov identifiers: NCT01926444 and NCT02276768).

Acute damage to the lining of the stomach can be induced by certain drugs (such as NSAIDs), stress and gastric ischaemia–reperfusion. H_2S is an important mediator of gastric mucosal defence; that is, the ability of the gastric mucosa to resist injury induced by endogenous and exogenous substances[139,140]. Pharmacological inhibition of H_2S synthesis increases the susceptibility of the stomach to injury, whereas H_2S donors (such as NaHS or diallyl disulfide) can protect the stomach from damage[139–141]. The underlying mechanism of the cytoprotective action of H_2S probably involves its ability to inhibit leukocyte adherence to the vascular endothelium[142] — a key event in the pathogenesis of NSAID-induced gastric mucosal damage[143]. H_2S can also trigger gastric and duodenal secretion of bicarbonate, which neutralizes excess mucosa-damaging acid[144,145]. Moreover, H_2S increases gastric mucosal blood flow, enhancing mucosal resistance to injury[139]. H_2S-releasing derivatives of NSAIDs have been shown to produce markedly less gastrointestinal damage than their corresponding parent NSAIDs[146–151]. True gastric ulcers, which penetrate into the submucosal layer of the stomach wall, take many days or weeks to heal, and clinically the healing of such ulcers can be accelerated to some extent by drugs that suppress gastric acid secretion. This healing is partially dependent upon H_2S, the synthesis of which is increased at the margins of the ulcer, where there is increased expression of CSE and CBS[152]. Oral administration of L-cysteine or H_2S donors (in this case, Lawesson's reagent or 4-hydroxythiobenzamide) to rats with gastric ulcers resulted in a significant acceleration of ulcer healing[152]. Whereas NSAIDs are known to retard the healing of gastric ulcers in humans and animals, H_2S-releasing NSAIDs have been shown to accelerate ulcer healing in mice[148]. Treatment with the H_2S donor NaHS has also been shown to significantly reduce the severity of reflux oesophagitis in rats, and inhibition of CSE in these animals led to the exacerbation of tissue injury[153].

NSAIDs also induce notable ulceration and bleeding in the small intestine, and there are no preventive or curative treatments available that have been proven to be effective for this potentially lethal condition[149,154]. Treatment with the H_2S donor diallyl disulfide can substantially reduce NSAID-induced enteropathy in rats[155], and H_2S-releasing NSAIDs induce negligible damage to the small intestine — even when such NSAIDs are co-administered with drugs such as aspirin and proton-pump inhibitors, which can markedly exacerbate NSAID-induced intestinal damage and bleeding[149].

H_2S appears to have a particularly important role as an anti-inflammatory, pro-healing molecule in the colon. In experimental colitis in rats, the local synthesis of H_2S is dramatically upregulated[18] at the sites of ulceration[156]. In this model, H_2S promotes the resolution of colonic inflammation and the healing of ulcers[18,156]. Inhibition of H_2S synthesis in healthy rats led to gastrointestinal mucosal inflammation, reduced expression of cyclooxygenase 2 and reduced synthesis of prostaglandin in the mucosa[18,91]. Administration of the H_2S donors Lawesson's reagent, NaHS or diallyl trisulfide to rats or mice with colitis resulted in faster resolution of inflammation and colonic tissue injury healing, as well as a downregulation of pro-inflammatory cytokine and chemokine expression relative to control animals[18,157].

Box 2 | **H_2S and bacterial–epithelial signalling**

The greatest source of hydrogen sulfide (H_2S) synthesis 'within' the human body may actually be an external source: the microbiota. In addition to contributing to the pool of H_2S, polysulfides and bound sulfane-sulfur[182,183], bacteria-derived H_2S has the capacity to influence many physiological and pathophysiological processes, particularly in the gastrointestinal tract. Bacteria-derived H_2S may contribute to several aspects of mucosal defence and repair, and certainly to the bioenergetics of gastrointestinal epithelial cells[184]. Conversely, H_2S donors appear to be able to influence the microbiota[155,185] — an effect that might be exploited in drug design to promote health.

Sulfate-reducing bacteria (SRBs) that produce H_2S reside in the gastrointestinal tract, where there are also sulfate-consuming bacteria. In a study of healthy individuals in the United States, approximately 50% of those studied had their gut colonized by SRBs, with *Desulfovibrio piger* — a member of the class of δ-Proteobacteria — being the primary H_2S producer[186]. Bacteria can also make polysulfides[187]. Just as it is now clear that circulating levels of H_2S in mammals were markedly over-estimated in the past[188], the concentrations of H_2S produced in the lumen of the gut were grossly overestimated for decades, contributing to the notion that H_2S was a primary driver of colonic inflammation and cancer[189–191]. At present, the balance of evidence suggests that H_2S is an important metabolic fuel for the epithelial cells that line the gastrointestinal tract and that seem to be particularly well adapted to this purpose, perhaps because of the hypoxic environment in which they reside[86,184]. The epithelium can therefore function not only as a physical barrier against potentially harmful agents that might pass from the lumen of the gut into the body, but also as a metabolic barrier, oxidizing bacteria-derived H_2S and, in doing so, generating ATP[151]. Bacterial H_2S can also stimulate the proliferation of gastrointestinal epithelial cells, and may contribute to the repair of any damage to the epithelial layer[192].

Conversely, the microbiota can be the target of actions of H_2S. Studies in rodents have demonstrated that intestinal microbiota in the colon form linear biofilms that appear to promote harmonious coexistence of the bacteria with the gastrointestinal mucosa[185]. When the mucosa is inflamed, the production of mucus is decreased and microbiota biofilms become fragmented. Delivery of H_2S into the colon promoted the resolution of inflammation, increased production of mucus, and restored normal biofilm structure, and this biofilm restoration was accompanied by reduced growth of planktonic bacteria[185]. In another study, administration of an H_2S donor to rats was shown to cause profound shifts in the microbiota, correcting the detrimental dysbiosis that had been triggered by the chronic administration of a nonsteroidal anti-inflammatory drug[155].

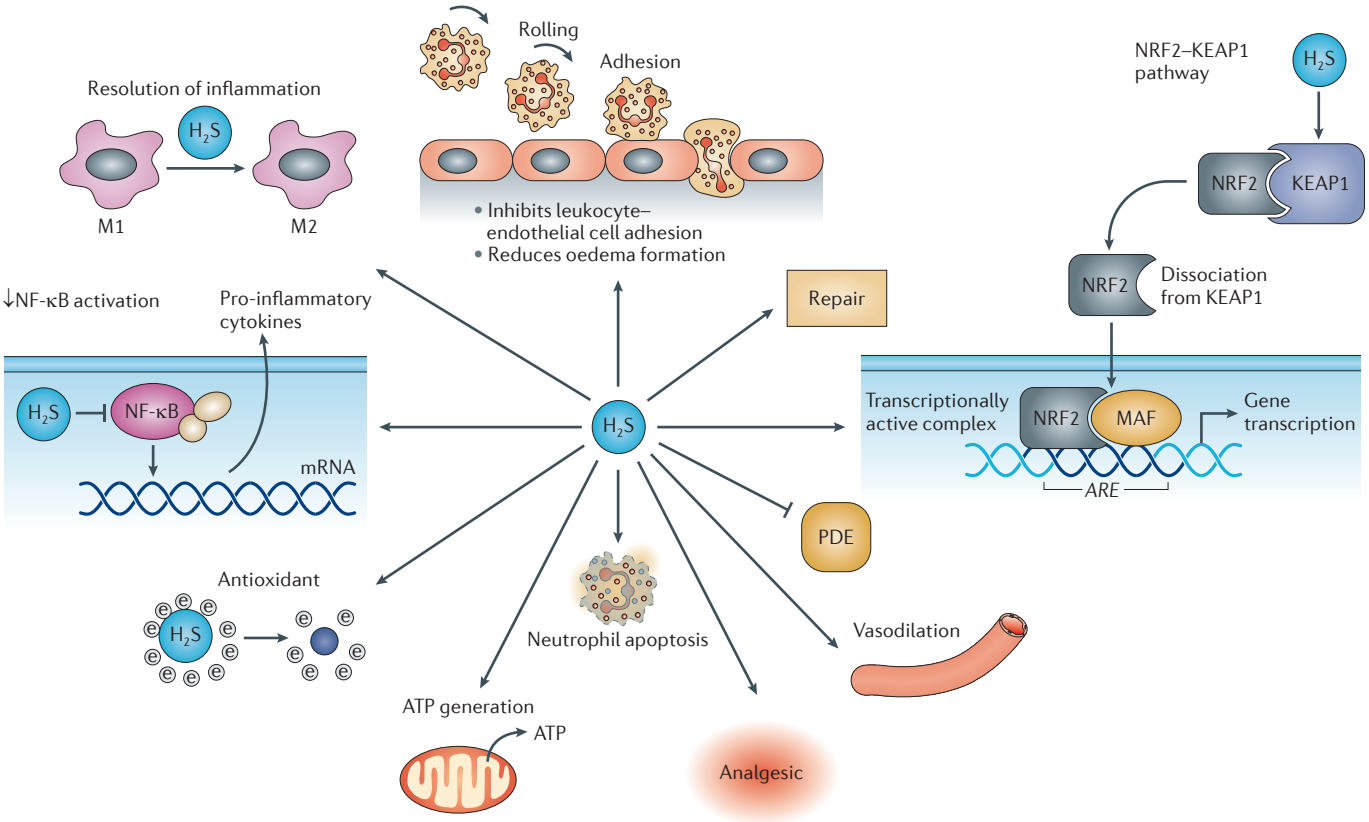


Figure 3 | **Anti-inflammatory and cytoprotective targets of H_2S.** Hydrogen sulfide (H_2S) is thought to act through several pathways, some of which are illustrated above, to reduce inflammation and protect tissues from injury (such as ulceration in the gastrointestinal tract). H_2S can suppress leukocyte adherence to the vascular endothelium, leukocyte extravasation and consequent formation of oedema. It can substitute for oxygen in driving mitochondrial respiration, thereby attenuating oxidative-stress-related tissue injury. The ability of H_2S to inhibit the activity of phosphodiesterases (PDEs) can contribute to its ability to relax vascular smooth muscle, resulting in enhanced blood flow. Resolution of inflammation can be enhanced by H_2S through actions such as the promotion of neutrophil apoptosis, and driving macrophage differentiation towards the M2 (anti-inflammatory) phenotype. H_2S can modulate the activity of a number of transcription factors: it inhibits nuclear factor-κB (NF-κB), leading to a reduced production of pro-inflammatory cytokines, and sulfhydrates Kelch-like ECH-associated protein 1 (KEAP1), which then releases active nuclear factor erythroid 2-related factor 2 (NRF2), resulting in increased expression of antioxidant-response elements (AREs). Increased production of H_2S occurs around sites of damage, such as around ulcers in the gastrointestinal tract, and can accelerate the healing of such damage via the stimulation of angiogenesis. Anti-nociceptive effects of H_2S have also been demonstrated. Adapted from REF. 193, American Physiological Society.

Impaired colonic H_2S synthesis, as observed in rats with hyperhomocysteinaemia, was associated with marked exacerbation of colitis, which could be reversed by administration of the H_2S donor diallyl disulfide[158]. IL-10-deficient mice, which spontaneously develop colitis, exhibited a similar defect in colonic H_2S production that could be reversed by administration of IL-10 (REF. 158). Thus, there is a substantial body of evidence suggesting that the anti-inflammatory or pro-resolution effects of H_2S are mediated to a large extent via downregulation of the expression of a range of pro-inflammatory cytokines (for example, IL-1β, TNF, interferon-γ (IFNγ), IL-12 and IL-23), although the expression of IL-10 is either spared or increased[147,157,158].

As discussed further below, several studies have demonstrated notable chemopreventive effects of H_2S-releasing drugs in animal models of colon cancer. Conversely, an upregulation of the expression of CBS, and the accompanying increase in H_2S production, have been suggested to play a key part in colonic tumour growth, in part by driving angiogenesis[26].

Human genetic diseases linked to H_2S-generating enzymes. Mutations in the human *CSE* gene can cause hereditary cystathioninuria and hypercystathioninaemia[159,160]. Similarly, inborn errors in the *CBS* gene are associated with the human hereditary diseases hyperhomocysteinaemia and homocystinuria[161]. As well as causing higher levels of cystathionine or homocysteine in the blood and urine, these diseases are associated with systemic inflammation, cardiovascular complications and damage to other organs. Conversely, overexpression of the *CBS* gene is a major cause of human Down syndrome, which is characterized by low levels of homocysteine in

the blood[162]. In addition, intronic polymorphisms and nonsense mutations in *MST* have been suggested to be responsible for a rare inheritable disorder known as mercaptolactate-cysteine disulfiduria[163].

H_2S-based therapeutics

An appreciation of the physiological and pathological importance of H_2S has been followed very quickly by attempts to develop novel therapeutics that aimed to deliver H_2S, or to suppress its endogenous production (TABLE 1). Furthermore, the impetus for the founding of Ikaria Therapeutics was the finding that H_2S given via inhalation could induce a state of 'suspended animation' in mice[164] by markedly reducing the metabolic rate. This company attempted to develop Na_2S for critical-care applications, such as for the reduction of myocardial injury following infarction[165]. However, in 2011, two clinical trials (ClinicalTrials.gov identifiers: NCT01007461 and NCT00858936) were halted, and Ikaria Therapeutics does not appear to be pursuing H_2S-based therapeutics at present.

Over the past decade, several other companies that focus on exploiting the potent anti-inflammatory and cytoprotective actions of H_2S have been founded (TABLE 1). Antibe Therapeutics, CTG Pharma and Sulfhydris have all developed H_2S-releasing derivatives of a number of drugs, with a major focus on NSAIDs[148–150,157]. The main targets for these drugs are pain and inflammation, with the primary benefit of H_2S release being the reduction in the gastrointestinal ulceration that is normally caused by NSAIDs. (However, CTG Pharma and Sulfhydris no longer appear to be active.)

The lead drug of Antibe Therapeutics (ATB-346; a naproxen derivative; TABLE 1) is being developed for the treatment of osteoarthritis, and has completed Phase I clinical trials in healthy volunteers (see the Antibe Therapeutics press release for further information). In animal studies of disorders such as adjuvant-induced arthritis, the drug produced anti-inflammatory effects that were comparable to those achieved with equimolar doses of the parent drug[148,149]. However, unlike the parent drug, ATB-346 produced negligible damage in the gastrointestinal tract, even at very high doses and in animals with impaired mucosal defence[148,149,166]. Naproxen was selected as the base NSAID, because it is the only member of the NSAID class that does not significantly increase the risk of serious cardiovascular events such as myocardial infarction and stroke[167]. In a Phase I clinical trial, ATB-346 administration to healthy subjects in doses escalating from 25 mg to 2,000 mg did not produce any notable adverse events, or any irregular cardiovascular, renal, haematological or hepatic effects. Antibe Therapeutics has additional drugs in preclinical development for treating acute pain (such as that associated with gout or sports injuries) and for veterinary pain and inflammation, as well as a novel anti-thrombotic drug that exhibits greatly increased gastrointestinal safety (TABLE 1).

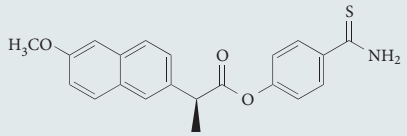
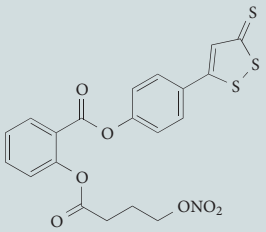
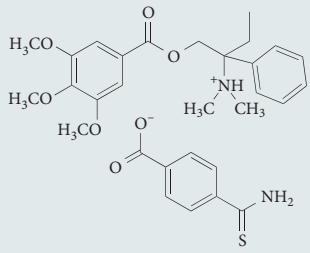
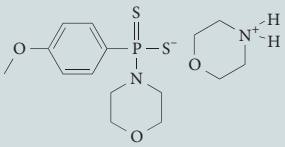
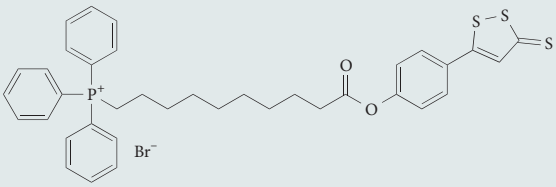
H_2S-releasing NSAID derivatives are also the principal focus of drug development efforts by a group at the City University of New York, USA. However, the main therapeutic application for these drugs, which have an NSAID linked to both a NO-releasing moiety and an H_2S-releasing moiety, is the treatment and chemoprevention of cancer. As well as being effective in *in vitro* and animal models of various cancers, these compounds exhibit significantly reduced adverse gastrointestinal effects[168,169]. The lead drug of this group is NBS-1120 (TABLE 1).

GIcare Pharma is attempting to exploit the reported ability of H_2S to reduce visceral pain[39,134,136]. Its lead drug, GIC-1001, is a salt — the counter-ions being thiobenzamide and trimebutine. Trimebutine is an opioid anti-spasmodic that has been used to treat a range of gastrointestinal conditions, including irritable bowel syndrome, for over 40 years. Shown to be safe in a Phase I clinical trial (ClinicalTrials.gov identifier: NCT01738425), this drug is now in Phase II clinical trials as a pre-colonoscopy analgesic (ClinicalTrials.gov identifier: NCT01926444 and NCT02276768). The rationale for this is that sedation during endoscopy is a routine practice, but can also be expensive and inconvenient. The use of GIC-1001 as pre-colonoscopy analgesic is proposed to reduce or even remove the need for sedation, possibly allowing the patient to leave hospital sooner, and translating to considerable savings for both the patient and the health-care provider. Trimebutine has been used as a treatment for irritable bowel syndrome[170], raising the possibility that the use of GIC-1001 may be extended to that indication in the future.

Several H_2S-based therapeutics target disorders that are characterized by oxidative stress and associated tissue injury. For example, largely on the basis of research by Elrod and colleagues[171], SulfaGENIX (in New Orleans, Louisiana, USA) is developing zerovalent sulfur (SG-1002) as a medicinal food, with the initial aim of targeting heart failure. Preclinical studies in relevant animal models of heart failure have confirmed that SG-1002 is effective in decreasing infarct size, improving cardiac function, increasing angiogenesis, decreasing inflammation and downregulating oxidative stress after infarction. A Phase I trial of SG-1002 (ClinicalTrials.gov identifier: NCT02278276) evaluated doses of 200 mg, 400 mg and 800 mg per day in healthy volunteers and reported dose-dependent increases in plasma H_2S levels, and only minor adverse effects. A second study has been designed (ClinicalTrials.gov identifier: NCT01989208) to evaluate the ability of SG-1002 to elevate plasma H_2S levels (in effect, reversing the defects in circulating H_2S) and to reduce markers of oxidative stress and heart failure.

Reducing oxidative stress is also the foundation of a series of novel compounds developed by Wood, Whiteman and colleagues at the University of Exeter, UK. Their compounds comprise an H_2S-releasing group linked to a mitochondrion-targeting group. Mitochondria have a critical role in determining whether a cell survives or dies[81] and, as discussed, H_2S can confer benefits to mitochondria by acting as an electron donor and downregulating the antioxidant-response pathway. The University of Exeter has patented (WO2013045951 A1) such H_2S-releasing compounds for the treatment of humans, animals or plants, and the authors propose that the compounds would be useful for the treatment of disorders such as hypertension and haemorrhagic shock,

Table 1 | **H_2S-based therapeutics in development**

Institution (location)	Structure	Clinical indications	Lead drug	Comment	Stage of development
Antibe Therapeutics (Toronto, Ontario, Canada)	 ATB-346	Osteoarthritis	ATB-346	Naproxen derivative	Phase I
		Acute pain	ATB-352	Ketoprofen derivative	Preclinical
		Veterinary (pain)	ATB-338	Diclofenac derivative	Preclinical
		Thrombosis	ATB-350	Aspirin derivative	Preclinical
City University of New York (New York, USA)		Cancer	NBS-1120	Aspirin derivative	Preclinical
Glcare Pharma (Montreal, Quebec, Canada)		Colonic pain	GIC-1001	Trimebutine salt; licensed from Antibe Therapeutics	Phase II for analgesia during colonoscopy*
National University of Singapore (Singapore)		Hypertension, inflammation, cancer	GYY4137	Slow-releasing H_2S donor	Unknown
Sova Pharmaceuticals (La Jolla, California, USA	No structure available	Pain, metabolic disorders	Unknown	Inhibitor of CSE activity	Unknown
SulfaGENIX (New Orleans, Louisiana, USA)		Oxidative stress	SG-1002	Polyvalent sulfur	Phase II for heart failure‡
University of Exeter (Exeter, UK)		Inflammation, oxidative stress	AP39	Mitochondrion-targeted H_2S release	Preclinical

CSE, cystathionine γ-lyase; H_2S, hydrogen sulfide. *ClinicalTrials.gov identifiers: NCT01926444 and NCT02276768. ‡ClinicalTrials.gov identifier: NCT01989208.

as well as conditions characterized by inflammation and oedema[80]. AP39 is the most advanced of these compounds (TABLE 1). It has been shown to elevate H_2S levels within endothelial mitochondria, protect cells against oxidant-induced damage and prevent damage to mitochondrial DNA *in vitro*[80].

Another university-based drug development programme with an H_2S focus is that of Moore *et al.* at the National University of Singapore. This group developed the H_2S donor GYY4137, which is now widely used as a research tool to study the effects of H_2S. Compared with conventional H_2S donors, GYY4137 releases H_2S more slowly. It is not clear whether this compound is in development or whether it is just a prototype for an H_2S-based therapeutic. GYY4137 has been shown to exert antihypertensive actions in SHRs[172], and to reduce inflammation through its ability to reduce circulating levels of various pro-inflammatory cytokines and mediators[173].

In this regard, GYY4137 has been shown to be an effective anti-inflammatory agent in a murine adjuvant-induced arthritis model[174]. The current status of the development of an H_2S donor by this group is unclear, but a patent is held for the use of a slow-releasing H_2S donor for the treatment of cancer (WO2014018569 A1).

Sova Pharmaceuticals is a company that was co-founded by Snyder, and is developing inhibitors of CSE for respiratory and metabolic disorders, as well as for diseases characterized by pain and inflammation, such as osteoarthritis and rheumatoid arthritis. The premise for this programme is that overproduction of H_2S contributes to the pathogenesis of several diseases — in particular, neuropathic and neurodegenerative diseases — as well as to inflammation and inflammatory pain. The current status of drug development by Sova Pharmaceuticals is unclear; however, the company filed a patent for CSE inhibitors in July 2013.

There are also several therapeutics on the market that were not developed specifically as H_2S-releasing drugs, but that could contribute to beneficial effects by releasing H_2S. For example, anethole trithione (sold under a number of brand names, including Sialor (Pendopharm) and Sulfarlem (EurekaSante)) is a drug that has been used for decades for treating dry mouth. Anethole trithione can generate H_2S, and is the same moiety that has been incorporated into H_2S-releasing drugs (for example, NBS-1120 and AP39 in TABLE 1) for preventing cancers, reducing oxidative stress and attenuating inflammation. Whether or not H_2S contributes to the desired clinical effects of anethole trithione is not clear. Like anethole trithione, oltipraz is a member of the dithiolethione class, and is used as a schistosomicide; however, it is not clear whether H_2S release contributes to its mechanism of action.

Zofenopril (Menarini) is an inhibitor of angiotensin-converting enzyme (ACE), and Bucci *et al.*[175] demonstrated that a large component of the anti-hypertensive effects of this drug occur independently of ACE inhibition, and are instead attributable to the H_2S released by this drug. *N*-acetylcysteine is a mucolytic drug used in the treatment of cystic and pulmonary fibrosis and also as an antidote for acetaminophen-induced liver damage. This drug can also generate H_2S, and has been shown to elicit marked anti-inflammatory effects in rodents[142], although roles for H_2S in mediating its above-mentioned clinical effects have not yet been established.

Future directions

Given the ubiquitous nature of H_2S, it is not surprising that it has important roles in a wide range of physiological and pathophysiological processes. The speed with which many H_2S-based therapies have been developed is a reflection of the excitement that this unique mediator has ignited and of the promising data that have been generated in preclinical and early clinical testing. The novel therapeutics that have been developed thus far offer the promise of increased efficacy, reduced toxicity, or both, compared with existing therapies. They range from very simple (for instance, zerovalent sulfur) to quite sophisticated approaches (such as H_2S release targeted to specific organelles).

In the near future, it is likely that organ-specific delivery of H_2S will be achieved, and with that, the possibility of disease-specific H_2S donors. It is also likely that the development of pH-, oxygen-, and free radical-sensitive donors will further facilitate the selective delivery of H_2S. Agents that could selectively activate the different H_2S-generating enzymes (namely, CSE, CBS and MST) are another intriguing possibility. Progress in the H_2S field has so far been hampered by the lack of availability of selective inhibitors of the various enzymes that contribute to the synthesis of this gasotransmitter. With enzymes such as CSE being identified as potential therapeutic targets, there will be increased motivation to develop potent and highly selective inhibitors that will hopefully be of value in research as well as for diagnostic and therapeutic applications.

As drugs that release H_2S or modulate its synthesis progress through the development path towards widespread clinical use, there will be a growing need to better understand the mechanisms of action of H_2S, and to be able to accurately monitor levels of H_2S *in vivo*. In addition to characterizing the effects of H_2S in various tissues and organs, attention will need to be paid to the potential impact that modulation of H_2S levels has on the microbiota (BOX 2), and in turn, the influence of any such changes on health and disease.

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Competing interests statement
The authors declare competing interests: see Web version for details.

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