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REVIEW

Hydrogen Sulfide Donors in Research and Drug Development

Zhi Jian Song,[a] Mei Ying Ng,[d] Zheng-Wei Lee,[d] Weilu Dai,[b] Thilo Hagen,[d] Philip K. Moore,[c] Dejian Huang,[b] Lih-Wen Deng*[d] and Choon-Hong Tan*[a]

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Hydrogen sulfide (H_2S) has recently emerged as an important biological gasomediator as a result of numerous insightful studies. The use of H_2S releasing compounds has attracted much attention as they can exert crucial effects on a wide range of cellular signaling processes. Some of these effects are potentially exploitable in terms of anti-inflammatory and anti-tumor effects, as well as precise ion-channel regulation, cardiovascular protection and oxidation resistance. Unfortunately, the potential therapeutic effects of H_2S are controversial due to conflicting published results regarding its effects on cellular activities arising, perhaps in part, from the use of different H_2S donors. Therefore it is essential to review the most commonly used H_2S releasing compounds, some of which are currently in clinical trials along with their associated *in vitro* and/or *in vivo* biological effects.

1 Introduction

Hydrogen sulfide (H_2S) is a colourless and poisonous gas which can be considered an environmental hazard when released as a by-product in industrial activities, as well as a metabolic hazard when individuals are exposed to concentrations sufficient to trigger undesirable physiological responses.[1] Interestingly, in the past decade, H_2S has been shown to have an important role as a novel biologically active gas, termed a gasomediator. Like the predecessors in this group of gasomediators, including nitric oxide[2] and carbon monoxide,[3] H_2S exerts fine regulatory roles to control a wide range of intracellular signaling processes.

H_2S is synthesized naturally in mammalian systems from L-cysteine (or 3-mercaptopyruvate) through four independent enzymatic pathways which are catalyzed directly or indirectly by endogenous enzymes such as cystathionine-γ-lyase (CSE), cystathionine-β-synthetase (CBS), cysteine aminotransferase (CAT), 3-mercaptopyruvate (3-MST) and cysteine lyase (CL).[4-6] L-cysteine required for this synthesis is usually derived either from dietary sources, through protein hydrolysis or from the L-methionine trans-sulfuration pathway. Not surprisingly, H_2S in mammalian systems is under tight dynamic regulation such that, at steady state cellular concentrations, it can exert important biological effects. As demonstrated in rat tissue homogenates by Doeller *et al.*, a trace amount of sulfide produced in the range of about 1-10 pmole per second per milligram protein is sufficient to execute fine roles in cellular functions.[7] This physiological effect was attributed to the key intermediate, H_2S.[8-10]

This paradigm shift in the therapeutic potential of H_2S was attributed to recent valuable work from the scientific community, shedding light on the desirable effects of the gasomediator on several physiological regulatory processes. For instance, a recent study revealed that H_2S may be very useful in the treatment of inflammation.[11] A large amount of evidence has shown that H_2S can act as an anti-inflammatory agent.[12,13] This effect relies on its ability to inhibit leukocyte adherence at the leukocyte-endothelium interface, possibly by increasing the expression of ICAM-1 (Intercellular Adhesion Molecule 1)[14] or by inhibiting the transcription of a cocktail of inflammatory genes through regulation of the activity of NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells).[12, 14] However, some studies also found that H_2S has pro-inflammatory effects[15] by raising plasma TNF-α (tumor necrosis factor-α) concentration and reducing lung and liver MPO (myeloperoxidase) activity.[16] With respect to cell proliferation studies, H_2S was shown to regulate protein kinases such as p38 mitogen-activated protein kinase and trigger cell apoptosis.[17] Some reports also pointed out H_2S donors displays anti-tumour effects, although the relevant mechanism remains uncertain at the moment.[18] Therefore, more and more H_2S donors have been developed for cancer research and therapy.

In the study of ion channels, Tang *et al* demonstrated that H_2S can open vascular smooth muscle cell ATP sensitive K^+ channels by regulating Ca^{2+} channel function.[19] The ability of H_2S to activate ion channels was extensively studied and H_2S was found to play a role as a gaseous signaling molecule in the central nervous system.[20] Based on the effect of H_2S on neuro-inflammation and the Ca^+ ion channel, more potential applications such as Alzheimer's disease[21] and Parkinson disease[22] therapy have been explored. Moreover, H_2S was also found to be potentially useful in treating vascular diseases,[23-26] such as hypertension and myocardial infarction,[5] as well as thrombus formation.[27] Therefore, some H_2S donors were developed as cardiovascular drugs and anti-thrombotic agents. On the other hand, H_2S can act as a radical and oxidant scavenger,[28,

[29] which is crucial in healthcare and cardiovascular protection.

Table 1 Summary of inorganic H_2S releasing compounds

Donor	Experiment	Effect	Result/Comment	Ref
NaHS	Ovalbumin-treated rats	Anti-inflammatory	H_2S exhibits anti-inflammatory effects and inhibits asthma pathogenesis remodeling *via* the CSE/H_2S pathway.	30
NaHS	Ovalbumin-induced acute lung injury	Anti-inflammatory	NaHS decreases inflammatory cytokines such as IL-6 and IL-8 levels but increases anti-inflammatory cytokine IL-10 levels.	31
NaHS	Lipopolysaccharide-induced inflammation	Anti-inflammatory	NaHS inhibits p38 mitogen-activated protein kinase and probably acts as a neuroinflammatory drug.	32
NaHS Na_2S	Air pouch inflammation model in rat	Anti-inflammatory	H_2S inhibits leukocyte adherence in mesenteric venules, as well as leukocyte infiltration and edema formation in animal models.	33
Na_2S	Acute arthritis	Anti-inflammatory	Na_2S exhibits anti-inflammatory properties in acute arthritis model, but no difference is observed in terms of pain sensation.	34
Na_2S	Burn and smoke inhalation induce acute lung injury model	Anti-inflammatory Antioxidant	Na_2S increases anti-inflammation cytokine IL-10, decreases cytokine IL-1β and attenuates protein oxidation.	35
NaHS	Wistar rats total hepatic ischemia and reperfusion	Anti-inflammatory Antioxidant	NaHS have potential therapeutic functions in reducing myocardial and renal inflammation and oxidation in the THIR model.	36
NaHS	IFN-gamma-primed human monocytic cell line U937	Pro-inflammatory	H_2S may stimulate the generation of pro-inflammatory cytokines partially through the ERK-NF-k B signalling pathway.	37
NaHS	Lipopolysaccharide-induced inflammation in mouse	Pro-inflammatory	NaHS causes histological inflammation by increasing MPO activity and raising plasma TNF-α concentrations in the lung and liver.	16
NaHS	CLP-induced sepsis and sepsis-associated lung injury	Pro-inflammatory	H_2S upregulates substance P (SP) which contributes to lung inflammation and injuries. It can also regulate leukocyte activation and trafficking in response to inflamed tissues.	38, 39
NaHS	Acute pancreatitis and lung injury mice model	Pro-inflammatory	By measuring chemokines in pancreas and lung tissues, NaHS was found to potentiate inflammatory responses.	40
H_2S	Sprague-Dawley rats SMC	Ion channels regulation	H_2S is an endogenous vaso-relaxant factor that activates K_{ATP} channels and hyper-polarizes membrane potential of vascular SMCs.	41
NaHS	Rat Cerebral Arterioles	Ion channels regulation	NaHS can decrease the myogenic response in rat cerebral arterioles. This may be related to the endothelium and its relation to K_{ATP} channels.	42
NaHS	Type 1 cell isolate from neonatal rat pups	Ion channels regulation	H_2S can inhibit ion of background K channels depolarization and voltage-gated Ca^{2+} entry and affect mitochondrial function in chemoreceptor cells.	43
NaHS	Stomatal closure in *Arabidopsis thaliana*	Ion channels regulation	NaHS regulates stomata opening in the light and closure in the dark.	44
NaHS	Astrocytes *in vitro*	Ion channels regulation	Increase in Ca^{2+} influx into cells by inducing Ca^{2+} waves and regulating Ca^{2+} storage in cells.	45
NaHS	Jejunal circular muscle strips	Ion channels regulation	Inhibition of contractile activity of jejunal circular muscle by NaHS and this is partly mediated by K_{ATP}^{+} channels and MIC phosphatases.	46
NaHS	Tobacco suspension cells	Ion channels regulation	NaHS increases heat tolerance in tobacco suspension cell via Ca^{2+} transfer into cells across the plasma membrane and mediation of intracellular calmodulin.	47
H_2S (g)	Partial cardiopulmonary bypass perfusion sheep	Cardiovascular effect	H_2S gas can cause pulmonary vasoconstriction and systemic vasodilation in cardiopulmonary bypass perfusion sheep.	48
NaHS	Hypertension mice	Cardiovascular effect	Mice lacking H_2S exhibited hypertension and diminished vasorelaxation while intravenous bolus injections of NaHS reduced this effect.	49
NaHS	Tissues isolated from adult rats	Cardiovascular effect	NaHS alone can relax smooth muscles. However, in the thoracic aorta, the effect was greatly enhanced by NO at low concentration of NaHS.	50
NaHS	Male Sprague-Dawley rats	Neurogenic regulation	Nanomolar levels of NaHS can inhibit corporal relaxation but endogenous H_2S has negative effects on neurogenic relaxation.	51
NaHS	Male Wistar rats	Tissue differentiation	NaHS can significantly induce osteoclast differentiation to TRAP-positive osteoclast combined with LPS through activation of the TLR4 pathway.	52

Having seen the various important functions of H_2S, different types of compounds which can release H_2S were employed as a tool to study the mode of action of H_2S in animal physiology. In medical studies, some of the H_2S releasing kinetic information of these compounds was determined,[53] such as the H_2S releasing profile of ACS14 in plasma,[54] the H_2S releasing profile of NOSH-Aspirin in liver,[55] as well as the releasing profiles of dithiocarbamates,[56] NaHS, GYY4137,[57,58] and some new H_2S donors,[59-62] in solution. In respect to the stability of H_2S donors, natural sulfur derivatives from derived garlic, allicin, were found to be extremely unstable, rapidly decomposed into diallyl polysulfides in human blood.[63] Over 80% of diallyl disulfides (DADS) from the garlic consumption get metabolized into sulfate within 90 min. No DADS was detected within the first 24 hr in blood sample of individuals who consumed garlic, suggesting that the natural sulfur derivatives have a short half-life in human body system. A better understanding of the precise mechanism of action of H_2S-releasing compounds is required before these compounds can move forward into clinical trials. Many studies conducted to address this have generated much controversy due to the different properties of the compounds which were used in the experiments. Hence, in this review, we will focus on representative compounds and highlight the disparate biological effects of these compounds. The compounds are grouped into the following categories: inorganic molecules, naturally occurring molecules, anethole trithione derivatives and synthetic molecules.

This review will also provide a brief evaluation on how these H_2S releasing compounds are used in research and drug development.

2 Activities of Inorganic H_2S releasing compounds

NaHS and Na_2S are two well-known inorganic H_2S releasing compounds that are widely employed in the study of H_2S effects on animal physiology (Table 1). Notably, Ikaria has developed a sodium sulfide solution (Na_2S) (IK-1001) for intravenous injection, which has successfully completed a Phase 1 clinical trial.[64] Because of its ease of availability, many studies have been conducted using NaHS which releases H_2S readily in the presence of water. However, due to the rapid onset of release, the lifetime of NaHS is short, making it difficult to maintain a constant cellular concentration of H_2S over a prolonged period. As such, there is a challenge in using NaHS to mimic the endogenous rate of H_2S production in the body. Nevertheless, the use of such inorganic H_2S releasing compounds has contributed to several functional discoveries.

2.1 Anti- and pro-inflammatory activities

Despite the large number of studies carried out to date (Table 1), the role of H_2S as a physiological mediator of inflammation remains controversial.[65] Both the anti-inflammatory and pro-inflammatory effects of exogenous H_2S donors such as NaHS or Na_2S have been documented. Chen *et al.* demonstrated that exogenous administration of NaHS into the lungs in an asthmatic rat model alleviated airway inflammation and remodelling.[30] In a separate study using an acute lung injury model which was induced by smoke and burn inhalation, the authors also found that clinical grade Na_2S protected against acute lung injury.[35] In addition, Andruski *et al.* showed that intra-articular injection of Na_2S inhibited leukocyte-mediated inflammation of the knee joint.[34] In an oleic acid-induced acute lung injury model, NaHS showed anti-inflammatory effects by decreasing the levels of pro-inflammatory cytokines IL-6 and IL-8, and simultaneously increasing the level of anti-inflammatory cytokine IL-10 in the plasma and lung.[31,51] Taken together, H_2S potentially acts as an anti-inflammatory agent in a variety of inflammatory conditions.

However, some reports demonstrated contrasting conclusions. For example, H_2S was found to promote the synthesis of pro-inflammatory cytokines in the U937 monocyte cell line *via* the ERK-NF-κB cascade.[37] Furthermore, the pro-inflammatory effects of H_2S were also observed using an array of inflammatory models including acute pancreatitis,[65] endotoxic shock[16] and lung inflammation.[40] In particular, when mice were subjected to cecal ligation and puncture (CLP)-induced sepsis, it was observed that NaHS enhanced the expression of cell adhesion molecules such as ICAM-1, P-selectin and E-selectin, thus promoting neutrophil infiltration.[39] This was further supported by studies which showed that NaHS also increased the expression profile of pro-inflammatory cytokines.[31, 39] Similarly, in an acute pancreatitis model induced by cerulein, H_2S exerted pro-inflammatory effects through the pro-inflammatory mediator substance P.[15] Under such experimental conditions, H_2S seems to display pro-inflammatory effects. As such, the role of NaHS or Na_2S as an anti-inflammatory or pro-inflammatory agent depends largely on the experimental setup: the experimental model of choice, the H_2S concentration used and the H_2S releasing rate.

2.2 Ion channel activities

Both endogenous and exogenous gaseous H_2S are able to stimulate the opening of vascular smooth muscle ATP-sensitive K^+ ion channels. The direct functional consequence of this is the dilation of blood vessels.[41] As evidenced by electrophysiological studies using aortic and mesenteric smooth muscle cells in rats, NaHS increased K_{ATP} channel currents and hyperpolarized the membranes. This provided direct proof of the effect of H_2S on K_{ATP} channels. In addition, Buckler demonstrated that H_2S in K^+ channels led to membrane depolarization and voltage-gated Ca^{2+} entry, thereby exciting carotid body Type 1 cells.[43] However, this effect of H_2S on background K^+ channel activity is a consequence of the well-understood role of H_2S in the inhibition of electron transport as well as of oxidative phosphorylation. Since H_2S is the first identified gaseous molecule with the ability to regulate vascular smooth muscle K^+ channels, much attention and excitement has been drawn into this area of research. Interestingly, some groups discovered that H_2S also relaxes colonic smooth muscle and inhibits contraction of isolated porcine irides *via* the activation and opening of K_{ATP} channels.[66] However, the mechanisms of action underlying the relaxant effect of H_2S on various other types of smooth muscles are unclear.[67] A previous study confirmed that NaHS can induce relaxation of vascular smooth muscle by a novel mechanism involving activation of the myosin-light-chain phosphatase.[50] The precise mechanism of how H_2S regulates these channels remains to be defined.

2.3 Other activities

Cardiovascular protection is another important effect of H_2S. NaHS was proved to counter the constriction of blood vessels that results from an elevation in blood pressure by inducing vasodilatation. It is the first evidence that H_2S can decrease such myogenic responses in rat cerebral arterioles by just being a vasodilator. This effect is achieved through activation of K_{ATP} channels by H_2S.[42] Derwall *et al.* demonstrated that H_2S gas administration *via* extracorporeal membrane lung ventilation had dual effects in an anesthetized sheep model.[48] It caused pulmonary vasoconstriction while inducing systemic vasodilation. The authors showed that high concentrations of H_2S in the cardiopulmonary bypass circulation did not reduce metabolism although it affected pulmonary and systemic vasomotor effects. In a separate study, inhibition of endogenous H_2S production by cystathionine γ-lyase deletion resulted in hypertension in mice.[49] This provided direct evidence that H_2S functions as a physiological vasodilator and regulator of blood pressure. Similarly, exogenous H_2S application results in relaxation of vascular smooth muscles isolated from adult rats.[50] In particular, a low concentration of H_2S greatly improved NO-induced smooth muscle relaxation in the thoracic aorta. In another study of the effect of H_2S on cardioprotection, Hu *et al.* found that H_2S preconditioning results in a decrease in myocardial infarct size as well as an improvement of heart contractile function in isolated rat hearts.[68] The authors showed that the mechanism underlying the cardioprotective effects of H_2S involves activation of the extracellular signal regulated kinase (ERK1/2) and phosphatidylinositol-3-kinase (PI3K)/Akt pathways.

H_2S has also been shown to alleviate spatial memory impairment in a β-amyloid rat model of Alzheimer's disease, thus shedding light on the potential therapeutic advantage of H_2S in the treatment of Alzheimer's disease.[69] Memory improvements were also observed in a 6-OHDA-induced Parkinson's disease rat model.[22] Taken together, these findings show that H_2S can potentially be exploited to confer neuroprotective effects to counter neurodegenerative diseases.

H_2S can also act on the nervous system in a manner which involves non-adrenergic and non-cholinergic neurotransmission. Nanomolar concentrations of NaHS were shown to inhibit relaxation of the rat corpus cavernosum *in vivo*.[51] It is suggested that this effect is a result of an interaction between endogenously produced H_2S and nitroxyl.

Another report by Irie *et al.* revealed that NaHS exerts effects on osteoclast differentiation.[52] The authors showed that co-treatment with H_2S and lipopolysaccharide resulted in an additive effect on tartrate-resistant acid phosphate-positive osteoclasts formation. This effect was attributed to the synergism between H_2S and lipopolysaccharide on toll-like receptor 4 pathway activation.

3 Activities of Natural H_2S releasing compounds

Besides cysteine and cystine, an array of sulfur-containing natural products is also found in various plants, animals, fungi and bacteria (Figure 1, Table 2). The most familiar sulfur-containing natural products are allicin and ajoenes which are found in garlic. Others are found in animals, for example, ovothiol from sea urchin eggs and varacin from marine ascidiacea. An example of a microbial source of a sulfur-containing natural H_2S releasing product is leinamycin,[70] which is synthesized by *Streptomyces*.[71] Others are found in fungi, such as ergothioneine and lenthionine. These compounds have recently been investigated to determine their therapeutic potential as H_2S can be produced from these natural products[72], either *via* endogenous enzymatic and/or non-enzymatic pathways.[73] For example, diallyl polysulfides which are present in garlic and onion can produce H_2S upon interaction with cysteine in the human body.[74] As such, these natural H_2S releasing compounds represent a wealth of prospective antioxidant, antibacterial, antifungal and anticancer properties that can potentially be exploited.[74] Indeed, some preliminary studies using natural products as anti-oxidative, anti-thrombotic and anti-cancer drugs have recently achieved satisfactory results *in vitro*.

Table 2 Summary of natural H_2S releasing compounds

Donor	Experiment	Effect	Result/Comment	Ref
DAT	Phorbol ester induced tumor	Anti-tumor	DAT can activate AP-1 and enhance COX-2 expression by blocking JNK in mouse skin thus exhibiting anti-tumor effect on mouse skin carcinogenesis.	75
DATS and analogues	Hep G2 cell	Anti-cancer	DATS can induce H_2O_2 formation, increase caspase-3 activity and lower the thiol level in Hep G2 cells.	76
Leinamycin	Defective mammalian cell line	Anti-tumor Antibiotic	Leinamycin can affect the NER (nucleotide excision repair) and BER (base excision repair) pathways, therefore helping to repair DNA damage.	77
Varacin	Human colon tumor	Anti-cancer	Varacin exhibits cytotoxicity towards the human colon cancer HCT 116 and toxicity towards the CHO cell line EM9 versus BR1.	78
Ajoene analogues	Different human cancer cell lines *in vitro*	Anti-cancer	Activity enhancement was observed, especially *p*-methoxybenzyl end group compound, which showed very good activity and a modest selectivity.	79
Z-ajoene	Human Cell line MCF-7, KB, Bel7402, BGC 823	Anti-tumor	Z-Ajoene showed potent inhibition of tumor growth both *in vitro* and *in vivo* because of the interaction with microtubule and it exhibited antimitotic properties.	80
SAMC	*In vitro* and *in vivo* colorectal cancer cells	Anti-cancer	SAMC inhibited the proliferation and metastasis of CRC cells effectively under both *in vitro* and *in vivo* conditions.	81
Ergothioneine	Neuronal hybridoma cell (N-18-RE-105)	Antioxidant	EGT can reduce H_2O_2 and ONOO induced oxidative damage. Moreover, it can act as non-toxic antioxidant *in vivo*.	82
Ergothioneine	Human erythrocytes *in vitro*	Antioxidant	These results show that human erythrocytes do take up ergothioneine; however, the GSH results do not support an antioxidant role for ergothioneine in erythrocytes. It also used for to scavenge peroxynitrite.	83
Ovothiol/ Trypanothione	Trypanosomatids and Sprague–Dawley rats model	Antioxidant	Ovothiol A and trypanothione can act as a non-enzymatic scavenger of hydrogen peroxide.	84
Ovothiol	*Strongylocentrotus purpuratus* eggs	Antioxidant	Ovothiol is more effective at decomposing H_2O_2 at the concentration produced during fertilization compared to catalase.	85
Ergothioneine	*In vitro* red blood cell	Antioxidant Anti-inflammatory	EGT was taken up by red blood cells and exhibited significant anti-oxidation and anti-inflammatory effects.	86
Ergothioneine	Acid induced Inflammatory	Antioxidant Anti-inflammatory	EGT has a protective role on palmitic acid-induced cell death due to oxygen radical scavenge and free fatty acids induced inflammation.	87
Ergothioneine	Resident skin cells	Antioxidant DNA repair	EGT can prevent DNA damage from oxidant of H_2O_2 and enable repair of UV-irradiated DNA damage in cells.	88,89
Lenthionine Varacin	Bacterial species and *Candida albicans*	Anti-bacterial	Lentinus edodes mycelium culture fluid exhibits anti-bacterial activities towards *Streptococcus pyogenes*, *Staphylococcus aureus* and *Bacillus megaterium*.	90, 91
Garlic extract	*In vitro* platelet aggregation model	Anti-thrombotic	Anti-thrombotic activity of a variety of structures related to ajoene was examined and results shed lights on the molecular basis for anti-thrombotic activity.	92

3.1 Anti-cancer and anti-tumor activities

Some natural products have shown interesting attributes such as anti-microbial, hypolipidemic, anti-thrombotic and anti-tumor properties.[93] For instance, some H_2S releasing compounds isolated from garlic and durian such as diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS) exhibit promising bioactivity. However, the role of H_2S in these biological activities has not been conclusively proven. DATS was shown to significantly suppress 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cyclooxygenase-2 (COX-2) expression and tumor proliferation.[75] Iciek *et al.* studied the effects of DAS, DADS and DATS on Hep G2 cell proliferation and the associated sulfur metabolism. Comparing DATS with DAT and DADS, DATS showed the highest activity in inhibiting Hep G2 cell proliferation.[76] In further elucidating the mechanism of action of these compounds, the authors found that these garlic-derived sulfur compounds induced H_2O_2 formation, promoted caspase-3 activity and lowered thiol levels in Hep G2 cells, thereby contributing to the inhibitory effects on cell proliferation. In a separate study of leinamycin and its analogues, Szilagyi *et al*. demonstrated that such analogues display cytotoxic activity against HeLa tumor cells.[94] Another group showed that the possible mechanism of this bioactivity is through interference with DNA repair processes.[77] Specifically, leinamycin induces DNA cleavage through nucleotide excision repair (NER) and base excision repair (BER) pathways.

Varacin, a polysulfide natural product, was also shown to be cytotoxic against human colon carcinoma cells.[78] Epidemiological studies demonstrated that ajoene analogues also have significant anti-cancer effects.[79] The Z-ajoene isomer exhibits substantial anti-cancer and anti-tumor activities.[80] Similarly, *S*-allylmercaptocysteine (SAMC) was shown to inhibit the proliferation of colorectal cancer cells under both *in vitro* and *in vivo* conditions. In particular, SAMC was found to inhibit Caco-2, SW480 and SW620 cancer cell proliferation and xenograft tumor formation.[81] It was proposed that the anti-cancer effect of SAMC is attributed to enhanced apoptosis as well as necrosis of the cancer cells. Clearly, such natural H_2S releasing compounds show immense opportunities for potential anti-cancer and anti-tumor effects to be exploited for therapeutic purposes.

3.2 Antioxidant activity

Ergothioneine (EGT) is widely distributed in fungi to higher organisms, but is known to be synthesized only by non-yeast fungi, mycobacteria and cyanobacteria. This natural sulfur-containing product has long been studied as an antioxidant agent[95] as well as a radical scavenger[96] and it is known to release H_2S.[97] EGT was investigated as an antioxidant agent in a human neuronal hybridoma cell line (N-18-RE-105). In a study using hydrogen peroxide and peroxynitrite treated cells, EGT was shown to mediate the inhibitory effects on peroxynitrite-induced oxidative damage.[82] Numerous *in vitro* assays have also proven the antioxidant and cytoprotective capabilities of EGT against a wide range of cellular oxidative stress. However, *in vitro* erythrocyte studies resulted in different conclusion. Mitsuyama and May's report did not support for an antioxidant role of EGT in erythrocytes despite the uptake of EGT by red blood cells.[83] However, a recent study showed that EGT can also function as a very effective antioxidant in red blood cells.[86]

Ovothiol which is highly present in marine invertebrate eggs acts as an effective hydrogen peroxide scavenger.[74,84] The oxidized ovothiol can react with GSH *via* thiol-disulfide exchange.[98] Subsequent redox recycling of GSSG and GSH would then produce H_2S.[99-101] In fact, it was found that this natural product can scavenge hydrogen peroxide even more effective than catalase at the physiological concentration produced during fertilization.[85]

3.3 Other effects

Some of these aforementioned natural-occurring compounds were also investigated for their anti-bacterial activities. For instance, varacin and three of its derivatives were found to exhibit substantial antimicrobial effects.[90] In a study of the mushroom *Lentinus edodes* and its cultured extract, a marked antibacterial effect was also observed and this was attributed to lenthionine.[91]

In addition, Laurenza, Weijgand-Heller, *et al.* found that in palmitic acid-treated skeletal muscle cells, EGT can inhibit MAPK activities and interleukin-6 expression, thus also display anti-inflammatory effects.[86,87] A study using aged garlic extract also showed anti-inflammatory effects and prevented coronary atherosclerosis in clinical trials.[102]

Ajoene is another well-known H_2S donor[103], and along with its homologues were investigated for anti-thrombotic effects *in vitro*. Based on a screen of an extensive library of ajoene homologues, it was found that various structures, such as sulfur oxidation state, double bond position, disulfide bond position, sulfide atom quantity as well as allyl groups can affect platelet aggregation.[92]

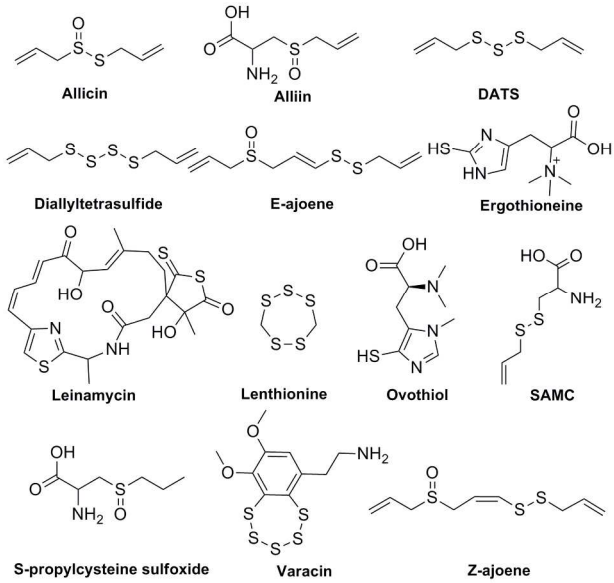

Figure 1 Structures of some natural H_2S donors

4 Activities of Anethole trithione derivatives as H_2S donors

Anethole trithione (ADT-OH, Figure 2) is a known H_2S releasing compound.[104] It is useful because it can be attached to various well-known non-steroidal anti-inflammatory drugs (NSAIDs)

such as aspirin and diclofenac. Some of these modified drugs are currently undergoing preclinical testing, namely: ACS-15 (CTG Pharma) for arthritis therapy, ATB-429 (Antibe) for anti-inflammatory activity in bowel disease and ATB-284 (Antibe) for use in the therapy against bowel irritable syndrome.

Table 3 Summary of anethole trithione derivatives as H_2S releasing compounds

Donor	Experiment Object	Effect	Result/Comment	Ref
ACS-15	lipopolysaccharide-induced Inflammation	Anti-inflammatory	H_2S-releasing diclofenac enhanced anti-inflammatory potency compared with parent drugs and greatly reduced the toxicity to gastropathy.	104
ATB-429	Colitis model of mouse	Anti-inflammatory	ATB-429 significant reduced granulocyte infiltration and reduced the expression of inflammatory cytokines/chemokines and showed enhance mesalamine activity.	105
NBS-1120	HT-29 human colon cancer cells xenograft model of tumor	Anti-cancer Anti-tumor	Suppressed the growth of cancer cells with 9000-fold more efficiency than the parts. A reduction of 85% the tumor volume was also observed.	55
HS-Sulindac HS-Ibuprofen HS-Naproxen HS-Aspirin	Colon, breast, pancreas, lung, prostate, and leukemia cancer cell lines	Anti-cancer	All of the HS-NSAIDs exhibited stronger suppression of cancer cell lines proliferation as compared with traditional NSAIDs. The IC50s were 28-3000 folds lower than the control.	106
HS-ASA	MDA-MB-231 cancer cell line and tumor xenografts in mice	Anti-cancer Anti-tumor	The HS-ASA regulated NF-kB and TrxR activity and induction of ROS thus suppressed the growth of cancer cells.	107
ACS 15	Cancer caused osteoclast *in vitro*, and osteolysis *in vitro*	Anti-cancer Anti-tumor	S-diclofenac derivatives suppressed breast cancer cell which provide supporting for osteoclastogenesis thus prevent osteolysis.	108
NOSH-1,2	Colon, breast, pancreas, lung, prostate, and leukemia cancer cell lines	Anti-cancer	NOSH-1 exhibited very efficiency anti-cancer effect but not caused any cellular toxicity, especially for HT-29 (IC50 = 48± 3 nM).	109
ACS14/ACS1	Glu and BSO injury RGC-5 cells	Antioxidant Ion channel regulation	ACS14 found to be an effective neuro-protectant partly by regulating oxidation stress, stimulating GSH and opening K^+ channels.	110
ACS14/ADTOH/ACS21	Sprague-Dawley rats and 3T3 cell	Anti-thrombosis Reduce toxicity	The ADTOH derives act as a new anti-thrombosis agent. They found the H_2S releasing property can reduce gastric mucosa by increasing H_2S/GSH formation and affecting redox imbalance.	54
ACS14	Human or murine whole blood and platelet rich plasma	Anti-thrombosis	Inhibition of thrombus formation *in vivo* in small arterioles as well as in larger arteries.	111
ATB429	Colorectal distension induced post-inflammatory hypersensitivity	Antinociception Anti-inflammatory	ATB-429 down-regulated Colonic COX2 and IL-1β mRNA and spinal c-FOS mRNA expression and inhibited hypersensitivity of pain.	112

4.1 Anti-inflammatory activity

Some NSAIDs have been known to cause serious gastrointestinal and renal side effects.[113] Numerous strategies have been employed to counter this problem. Based on previous studies of NO, it is well-known that NO displays local gastric mucosal defence properties[114] and these properties have been exploited in the development of NO-releasing NSAID compounds. Due to the similar activities of H_2S and NO,[115] several H_2S-releasing NSAID derivatives were also developed in order to reduce damage to the gastric mucosa.[116] As expected, ACS-15 (*S*-diclofenac, ATB-337) releases H_2S slowly in liver homogenates, thus maintaining the plasma concentration of H_2S at a high level for a prolonged period of time. In a lipopolysaccharide-induced inflammatory model, ACS-15 causes gastric toxicity whilst reducing lung and liver myeloperoxidase activity which is indicative of reduced neutrophil infiltration in these organs.[104] Similar results were also reported in another study with ATB-429 (*S*-mesalamine). Fiorucci *et al.* emphasized that ATB-429 displays marked anti-inflammatory activity and *in vivo* potency as compared to mesalamine.[105] Based on a study of the pharmacological profile of *S*-aspirin (ACS-14 and ACS-21), the authors noted that it could reduce side effects on the gastric mucosa by regulating redox imbalance.[54] It also demonstrated high bioactivity and therapeutic safety in amyloid β plaque-induced inflammation,[117] thus shedding light on the potential therapeutic value of these compounds in the treatment of Alzheimer's disease.[117]

4.2 Anti-cancer and anti-tumor activity

Due to the synergistic effect on the inhibition of cyclooxygenases, some of the ADT-OH derivatives containing both a H_2S releasing functional group and NSAID were also investigated for anti-cancer properties. For example, NBS-1120 (NOSH-ASA) was observed to suppress HT-29 colon cell proliferation with IC_{50} values in the nanomolar range. In addition, in *in vitro* experiments NBS-1120 was used in a tumor xenograft model and an excellent anti-tumor effect was observed.[55] In addition, aspirin, sulindac, ibuprofen and naproxen coupled with ADT-OH were tested on various types of cancer cell lines.[106] The results showed that HS-aspirin was the most effective drug for most of the cancer cell lines tested. Furthermore, HS-aspirin[107] and ACS-15[108] suppressed cancer cell proliferation *in vitro* and significantly suppressed xenograft tumor growth. For compounds such as NOSH-1 and NOSH-2 with both H_2S and NO releasing properties, they were shown to exhibit anti-cancer effects in the HT-29 cell line.[109]

4.3 Anti-thrombotic activities

In addition to anti-cancer and anti-inflammatory activities, anethole trithione derivatives were also investigated as anti-thrombotic,[54] antioxidant[118] and neuroprotective[110] agents. Pircher *et al.* reported that ACS14 inhibited human or murine

blood platelet aggregation[111] and thus could potentially be used as an effective thrombus formation inhibitor *in vivo*. Distrutti found that ATB-429 acts as an anti-nociceptive agent due to activating K_{ATP} channels.[112,119]

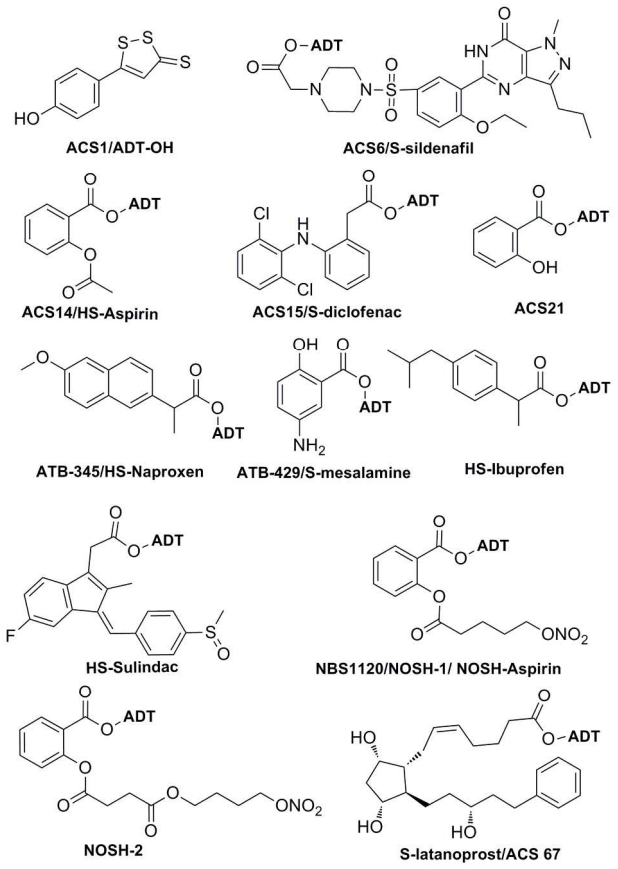

Figure 2 Structures of anethole trithione derivatives

5 Activities of Synthetic H_2S releasing compounds

In addition to the above-mentioned H_2S donors, there are a number of compounds that have been specifically designed by synthetic chemists to release H_2S (Figure 3). Compounds containing active P-S bonds such as Lawesson's reagent[120], GYY4137[57] and Phosphorodithioate[121] have been used as H_2S donors. As reported in the 6th European Congress of Pharmacology, thioamides can be hydrolyzed to release H_2S. To exploit this characteristic, thioamide compounds such as NOSH-3[109] and ATB-346[122] were investigated along with anethole trithione for anti-cancer activities. Recently, some arylthioamides emerged due to their remarkable vascular effects *in vitro* and *in vivo*.[60] Similarly, rhodanine and its derivatives contain the same dithiocarbamate ester. It releases H_2S with acids[56] or reducing agents.[123] They were developed as scaffolds in drug discovery.[124] For some other sulfide drugs like NOSH-4,[109] ACS86,[125] Perthiols[61] and dithioperoxyanhydrides,[126] the disulfide bond can react with thiol and reductive molecules in organisms to release H_2S. Cys-Act hydrogen sulfide donors are stable in the absence of cysteine[127], but can react with cysteine *via* NCL (native chemical ligation) to release H_2S.[44] Also, new controllable H_2S donors have been reported, such as gem-dithiols,[62] thioglycine and thiovaline.[59] These synthetic H_2S donors have similar bioactivities such as anti-inflammatory effects, anti-cancer effects, opening of Ca^{2+} and K^+ ion channels and regulatory effects on plants.

5.1 Smooth muscle relaxation

H_2S-releasing drugs can potentially be used in the treatment of cardiovascular disease. For example, GYY4137 has been shown to cause a slow relaxation of rat aortic rings.[98] It also functions as a vasodilator in perfused rat kidney. This compound has also been investigated as a myometrium regulatory agent to prevent preterm labour.[128] When myometrial tissues are exposed to a solution of GYY4137, relaxation of the uterus is observed. These studies suggest that synthetic H_2S releasing compounds have potential in the treatment of cardiovascular disease and the control of uterine contractility.

5.2 Anti-cancer activity

A number of synthetic H_2S releasing compounds have been shown to have anti-cancer activities. For instance, ACS32 (see Figure 3) was found to inhibit breast cancer cell proliferation. It also exhibits anti-osteolytic effects by inhibiting osteoclast formation and activity.[90] Thus, ACS32 may be a good candidate for the treatment of osteolytic bone diseases. NOSH-3 and NOSH-4, on the other hand, are aspirin derivatives that can also suppress cancer cell proliferation, but are less effective compared to the anethole trithione derivatives of aspirin.[109] GYY4137 was also found to exhibit anti-cancer effects in HeLa, HCT-116, Hep G2, HL-60, MCF-7, MV4-11 and U2OS cells and to reduce tumor growth in a mouse xenograft model.[58] Importantly, GYY4137 displayed only limited toxicity in normal human lung fibroblasts (IMR90, WI-38).

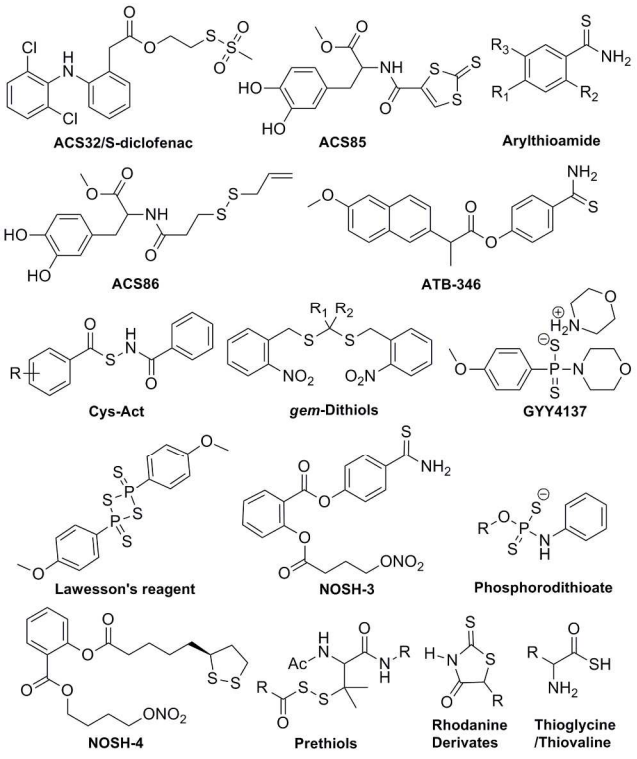

Figure 3 Structures of other synthetic H_2S donors

5.3 Anti-inflammatory activity

GYY4137 has been reported to have anti-inflammatory activities in a number of studies. For instance, the H_2S releasing compound was recently studied in an arthritis model[129] and an inflammatory joint model.[130] The results revealed a significant reduction in the levels of inflammatory mediators as well as oxidative stress. Similar anti-inflammatory activities of GYY4137 were observed on induction of endotoxic shock or inflammation by lipopolysaccharide in rats.[131] A separate study on the effect of GYY4137 on lipopolysaccharide-induced release of inflammatory mediators from macrophages provided evidence that inflammatory processes depend on both the H_2S concentration and its rate of release.[132]

A further H_2S releasing compound, ATB-346, was found to be as effective as naproxen and celecoxib in reducing inflammation and inhibiting cyclooxygenase activity. Importantly, ATB-346 did not cause significant gastric or intestinal damage in any of the models studied[133] and was 100-fold safer compared to the use of naproxen in healthy animals.[134]

Table 4 Summary of synthetic H_2S releasing compounds

Donor	Experiment Object	Effect	Result/Comment	Ref
GYY4137	Stomatal closure in *Arabidopsis thaliana*	Ion channels regulation	GYY4137 and NaHS can regulate stomatal opening and closure due to the effect of NO in guard cells.	44
GYY4137	Aortic rings and perfused rat kidney Anesthetized rat *in vivo*	Cardiovascular Protection	Low concentrations of H_2S acting on isolated rat heart did not have any direct effect on cardiac rate and contraction force but it can regulate vasorelaxation slowly and enduringly, thus resulting in a fall in blood pressure and reduce hypertension with no toxicity.	57
GYY4137	Human and rat myometrium	Ion channels regulation	GYY4137 can decrease myometrium contractions, reduce underlying Ca^{2+} transport and reduce oxytocin induced and K^+ related myometrium contractions activities.	128
ACS32	Cancer caused osteoclast *in vitro*, and osteolysis *in vitro*	Anti-cancer	*S*-Diclofenac derivatives suppressed breast cancer cells proliferation, which provided supporting evidence for osteoclastogenesis that prevents osteolysis.	108
NOSH-3 NOSH-4	Colon, breast, pancreas, lung, prostate, and leukemia cancer cell lines	Anti-cancer	All of the analogues exhibited anti-cancer effects: NOSH-3 is about 20-150 times less effective than NOSH-1, and NOSH-4 is about 1-17 times less effective than NOSH-1.	109
ATB346	Mouse model of colon cancer	Anti-cancer Reduce toxicity	The H_2S releasing NSAIDs suppressed colon cancer cells proliferation in a mouse model, and it also reduced gastro-intestinal toxicity.	122
GYY4137	Different human cancer cell lines *in vivo* and *in vitro*	Anti-cancer	GYY4137 significantly reduced HL-60 and MV4-11 tumor cells growth.	58
Rhodanine derivatives	Cellular DDX3 and HIV-1 replication	Anti-HIV	Rhodanine derivatives have inhibitory activities to cellular DDX3 (a valid anti-HIV target), thus exhibiting anti-HIV effects.	135
Rhodanine derivatives	*In vitro* antimicrobial	Antimicrobial	Rhodanine derivatives had strong antibacterial activity against the methicillin-resistant *Staphylococcus aureus* (MRSA).	136
Rhodanine derivatives	*In vitro M. tuberculosis* $H_{37}Rv$	Antimicrobial	A series of compounds exhibited good activity in inhibiting TB with MIC at μg levels. Rhodanmine derivatives may also be used as scaffolds for anti-TB agents.	137
GYY4137	Human articular chondrocytes and mesenchymal progenitor cells	Anti-inflammation	GYY4137 significantly prevents oxidative stress-induced cells from death and acts as a protector in inflammed joint by analysing the cell availability and the expression the CBS/CSE.	76
GYY4137	NCTC 2544 human keratinocytes	Anti-inflammation	H_2S can modulate endogenous NO and VEGF in human keratinocytes, thus exhibiting therapeutic effects for chronic inflammatory disorders of the skin.	138
GYY4137	Human synoviocytes, articular chondrocytes and rat models	Anti-inflammation	GYY4137 reduces pro-inflammatory factors and inhibits the activity of related enzymes.	129, 131, 132
ATB346	Compromised mucosal defence rats and healthy rats	Reduce toxicity Anti-inflammatory	H_2S releasing naproxen decreases gastrointestinal damage in impaired mucosal defence model and showed anti-inflammatory effects.	133, 134
ATB346	Rats with knee joint synovitis	Anti-inflammatory Anti-nociceptive	ATB-346 reduces the side effects of gastric mucosa damage but does not reduce the inflammation and hyperalgesia in rats.	139
ACS85 ACS86	BV-2 cells treated with $A\beta_{1-40}$ and Neurons isolate from rats	Nervous protection Anti-inflammation	ACS 85 and ACS86 may protect microglial cells against injury *via* the anti-inflammatory and antioxidant activities; potential for use in the treatment of neurodegenerative diseases.	125, 140
Thioamide	In vivo and noradrenaline treated rat aortic rings	Cardiovascular protection	The selected thioamide could dilate NA-treated blood vessels and reduce the systolic blood pressure *in vivo*.	60
Perthiols	Ischemia-reperfusion myocardial	Cardiovascular protection	The perthoils, which could release H_2S by regulating of thiols, exhibited cardioprotective effect in the murine myocardial injury model.	61

5.4 Other effects

H_2S releasing compounds have been shown to exert various other effects. For instance, a number of rhodanine derivatives were shown to have anti-bacterial activities against both Gram-positive and Gram-negative bacteria.[136] Rhodanine derivatives were also shown to exert anti-HIV activities by inhibiting the cellular ATPase DDX3.[135] They can also act as PDE4 (phosphodiesterase-4, related to immune cells and central nervous system cells) inhibitors,[141] anti-tubercular agents[137] and anti-

inflammatory drugs.[142] However, the wide variety of bioactivities observed may be non-specific to target many proteins.[124] In particular, their analogues have been shown to have activities against *Mycobacterium tuberculosis* at a very low minimum inhibitory concentration (MIC).[137] The roles played by H_2S in the above-mentioned reports need to be clarified. In addition, novel hybrid compounds containing both L-DOPA and H_2S donors inhibited the release of pro-inflammatory cytokines and nitric oxide from stimulated microglia and lowered amyloid beta-induced cytotoxicity.[118,119] Hence, these compounds have potential in the treatment of neurodegenerative diseases such as Alzheimer's disease[21] and Parkinson disease[22, 125].

6 Conclusions and Outlook

H_2S releasing compounds exhibit complex biological activities and exert multiple physiological effects. In addition to anti-inflammatory and anti-cancer activities, H_2S releasing compounds also have anti-oxidant effects and can regulate some cardiovascular functions through ion channels. However, H_2S donor development is still at an early stage even though H_2S has been studied for a few decades. Most of the drugs were studied in limited areas despite the wide regulatory functions of H_2S, so there is scope to unfold the other potential applications of different H_2S donors.

Inorganic salts of sulfide serve as easily accessible tools to study the biological roles of H_2S, but their acute release rate and low sustainability make them less ideal H_2S donors. Natural H_2S releasing compounds are useful as anti-tumor and antioxidant agents. On the other hand, anethole trithione is particularly interesting due to its ability to conjugate with NSAIDs to form safer and potentially better anti-inflammatory and anti-cancer drugs. Last but not least, a wide variety of new synthetic H_2S compounds reflects the great potential of H_2S donors with different releasing properties for widespread applications. Based on the scope of H_2S donors in application, effective building blocks such as polysulfide, anethole trithione, thioamide, disulfide, P-S bonds and some other activated sulfides have been evaluated. These sulfide units can combine to specific scaffolds for targeted therapy.

To fully harness the biological activities of H_2S, it is important to develop novel H_2S releasing drugs. Desirable H_2S donors should release H_2S slowly and consistently. They should also not contain any structures that have significant biological side effects or cause toxicity. In addition, the solubility of the H_2S donors needs to be carefully controlled to ensure a good pharmacokinetic profile. Finally, the donor should also have good stability in aqueous solution. The synthesis of H_2S releasing compounds with improved properties will help to move these donors towards clinical trials.

Notes and references

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a Division of Chemistry & Biological Chemistry, School of Physical & Mathematical Sciences, Nanyang Technological University, Singapore
b Department of Chemistry, National University of Singapore
c Department of Pharmacology, National University of Singapore
d Department of Biochemistry, National University of Singapore

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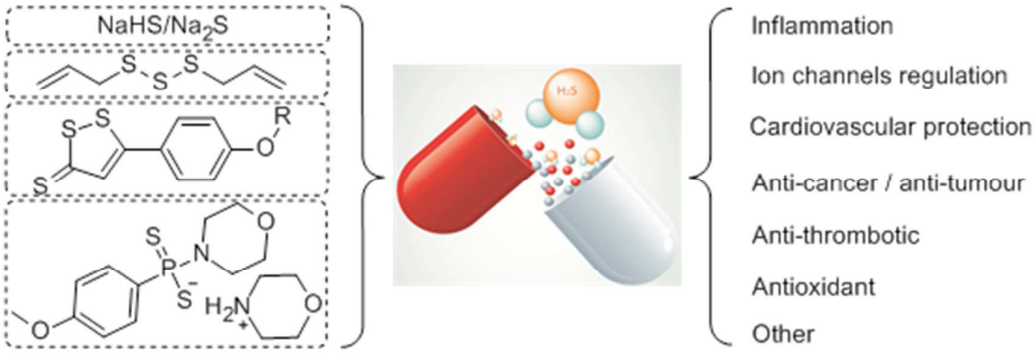
Hydrogen Sulfide Donors in Research and Drug Development

Zhijian Song,[a] Mei Ying Ng,[d] Weilu Dai,[b] Thilo Hagen,[d] Philip K. Moore,[c] Dejian Huang,[b] Lih-Wen Deng*[d] and Choon-Hong Tan*[a]



Abstract： This review summarized most of H_2S donors such as inorganic compounds, natural products, anethole trithione derivatives and synthetic compounds used in research and drug development. There special bioactivities provided us some effective strategies for antiphlogosis, cancer therapy, cardiovascular protection and so on.