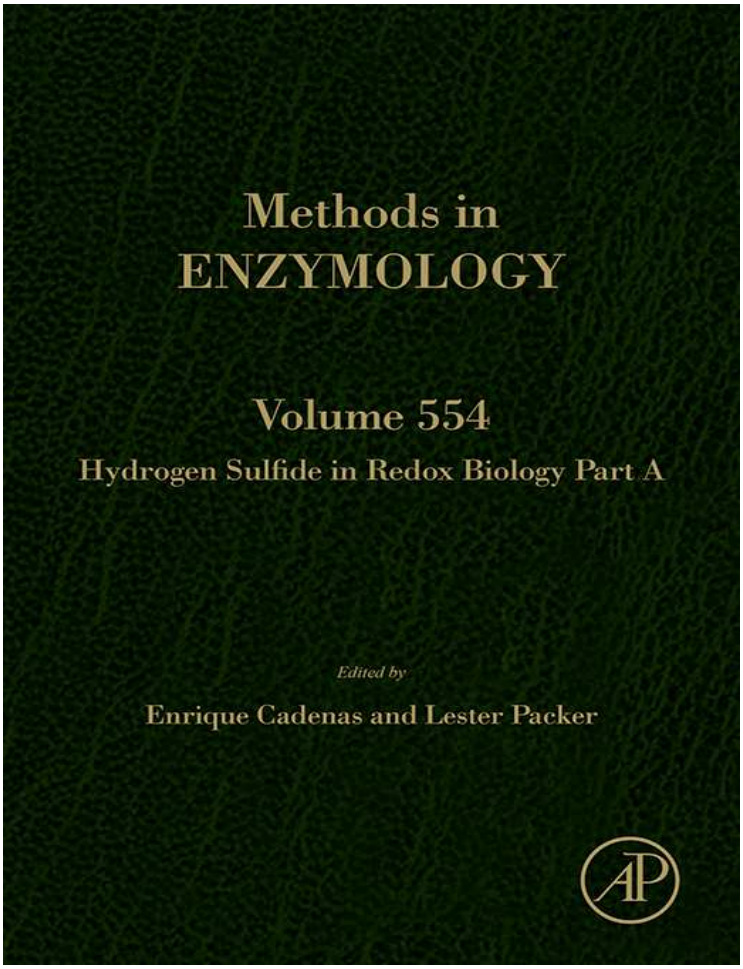

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CHAPTER NINE

GYY4137, a Novel Water-Soluble, H_2S-Releasing Molecule

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Abstract

Hydrogen sulfide (H_2S) is now recognized as the so called "third gasotransmitter" taking its place alongside nitric oxide and carbon monoxide. In recent years, H_2S has been reported to exhibit a diverse range of pharmacological effects in biological systems. Much of this evidence is derived from a combination of conventional pharmacological and genetic approaches coupled with the use of chemical compounds such as sodium hydrosulfide, a rapid H_2S releasing donor. Developments in the design of new drug entities which attempt to take into account physicochemical properties, targeting to specific cellular organelles, triggering of H_2S release upon specific chemical reactions in the cell, and controlling the release of H_2S over extended periods of time have been described. For most of these molecules, little or no work has been conducted to determine their biological activity or possible therapeutic effects. It is therefore not clear whether such molecules have therapeutic potential which highlights the need for further *in vivo* studies. One exception to the general rule is GYY4137 (morpholin-4-ium

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4-methoxyphenyl(morpholino) phosphinodithioate), a slow releasing H_2S donor, which has been evaluated for activity in a range of pharmacological models both *in vitro* and *in vivo*. GYY4137 was first reported to release H_2S and exhibit vasodilator activity over 5 years ago and, to date, GYY4137 is becoming increasingly employed as a pharmacological "tool" to explore the biological functions of H_2S.

1. INTRODUCTION

In recent years much attention has been focussed on elucidating the functional roles of the gaseous mediator hydrogen sulfide (H_2S) in biological systems (for reviews see, Guo, Cheng, & Zhu, 2013; Li, Rose, & Moore, 2011; Olas, 2014; Wang, 2012). It is now clear that biologically active quantities of H_2S are synthesized naturally by a number of cell types in a range of different tissues in mammals, nonmammalian species, and even plants. Moreover, evidence is growing that its principal role is to act as a signaling molecule bringing about a distinct set of physiological and biochemical effects.

Considerable emphasis has been placed on determining the role of this gas in normal physiological processes and, at the same time, whether changes in the homeostatic regulation of the H_2S system can trigger, contribute to, or exacerbate disease. A better understanding of the physiology and pathophysiology of H_2S will be key to the development of novel therapeutics based on the general principles of either, (i) reducing endogenous H_2S biosynthesis or, as will become apparent in this review, more likely, (ii) replenishing deficient production of this evanescent gas.

Much progress has been made in understanding the biology of H_2S in the last decade. Thus, without going into specifics which will be dealt with elsewhere in this volume, H_2S has been reported to play a part in a range of biologically fundamental processes which affect most cells/tissues including mitochondrial function, cytoprotection, inflammation, vascular perfusion, tissue preservation and repair, apoptosis, cell cycle regulation, and aging. Interestingly, many of these effects of H_2S in biological systems are not restricted to mammalian species. For example, biosynthesis of this gas has been detected in the tissues from organisms found across several taxonomic kingdoms (Calderwood & Kopriva, 2014; Clarke, 1953; Julian, Statile, Wohlgemuth, & Arp, 2002; Olson et al., 2008; Papenbrock, Riemenschneider, Kamp, Schulz-Vogt, & Schmidt, 2007). In the plant

kingdom, exogenous application of H_2S has been reported to increase crop yields, germination rates, influence root development, and alleviate heavy metal ion induced stress responses (Calderwood & Kopriva, 2014; Lisjak, Teklic, Wilson, Whiteman, & Hancock, 2013). Certainly, a considerable body of work now exists describing the functional roles of H_2S in bacterial, animal, and plant systems.

Much attention has, not surprisingly, been focussed on the mechanism(s) by which cells generate H_2S. By and large, the synthesis and production of this molecule is largely mediated *via* enzymatic routes. Recent evidence supports the liberation of H_2S from endogenous persulfides and polysulfide species (Greiner et al., 2013; Ida et al., 2014; Miranda & Wink, 2014). Intracellular levels of glutathione persulfide were quantified at over 100 μM in mouse brain leading to the hypothesis that exogenous H_2S is taken up primarily into the "persulfide pool." The biological equilibrium is hence heavily weighted in favor of persulfides which could be major sulfur transfer signaling molecules. The major biosynthetic origins for H_2S in mammals are *via* the catabolic activity of cystathione β synthetase (CBS, EC 4.2.1.22), cystathionine γ lyase (CSE, EC 4.4.1.1) on L-cysteine and 3-mercaptopyruvate sulfurtransferase (3-MST, EC 2.8.1.2) on 3-mercaptopyruvate. Worth noting is that additional systems are also likely involved in H_2S homeostasis yet their relative contribution in maintaining tissue H_2S levels remains largely unknown and, undoubtedly, other pathways responsible for H_2S biosynthesis and degradation will likely be discovered.

Perturbations in endogenous H_2S biosynthesis *via* one or other of these enzymatic pathways, may cause or play a part in disease states. For the most part, these disease states have been linked to loss of H_2S production due to reduced activity or expression of CSE, CBS, 3-MST, or a combination thereof. Although somewhat simplistic, a variety of conditions including hypertension, inflammation, atherosclerosis, sexual dysfunction and reperfusion injury, and perhaps also cancer, are associated with reduced H_2S generation or availability. Clearly, multiple pathologies and mechanisms underpin these diseases but, intriguingly, a lack of H_2S production seems to be at least one common thread. Much effort has therefore been expended to understand the processes whereby both the activity and the expression of CSE, CBS, and 3-MST are controlled. This work is ongoing and will help to unravel the complex biological significance of H_2S.

2. WHY SLOW RELEASING H_2S DONORS?

As noted above it is becoming increasingly clear that a number of disease states have been linked to what might be described as a state of "relative H_2S deficiency" in different cells, tissues and/or organs. As such, the expeditious use of H_2S donor molecules may, logically, provide a possible common therapeutic approach to a wide range of disease states by replenishing H_2S. A number of H_2S donors have been reported in recent years. In addition to H_2S gas itself, by far the most commonly used are the simple sulfide salts such as NaHS and Na_2S. The use of these salts has been widespread in the literature over the last decade and has undoubtedly added greatly to our understanding of the complex biological effects of H_2S. It could even be argued that the widespread availability of, for example, NaHS, which is both inexpensive to purchase and readily soluble in water, has been a crucial factor in creating the interest and giving impetus to researchers to study H_2S, especially so in the early days. However, a major concern with NaHS is that it does not allow for the controlled release of H_2S at rates that would be expected to mimic physiological conditions. Indeed, once solubilized, NaHS releases large amounts of H_2S spontaneously, i.e., a bolus effect. It is very unlikely that endogenous H_2S, made enzymatically in cells and tissues as detailed above, could ever be generated at the very high concentrations and over the very short time period, which is the case when cells or tissue are exposed to NaHS. One caveat in this argument is that the concentration of endogenous H_2S generated enzymatically within individual tissues or indeed within individual subcellular components inside the target cell is not known and will require the development of much more sensitive and selective methods to detect this gas than are currently available.

With this in mind, a consequence of the explosive H_2S release profile of NaHS is that the biological effects elicited by this, or like, compounds when introduced into a biological system (i.e., a conscious or anesthetized animal, cells maintained in culture or tissues in organ baths) reflects the pharmacological (perhaps toxicological) activity of H_2S and not its physiological role within that tissue/cell. As such, the use of compounds like NaHS, whilst valuable, may in some cases, obscure rather than illuminate, the biology of this gas. Clearly, using NaHS to probe for physiological roles of H_2S is problematic. One approach to overcome this shortcoming has been the development of organic molecules which slowly release H_2S at rates which are more akin to the rate expected from cells *in vivo* (Song et al., 2014).

A number of H_2S donors of different types are now available. Many of these have been designed with the specific intent of delivering controlled levels of H_2S gas to tissues. Of particular interest are a number of structurally divergent molecule classes encompassing H_2S releasing nonsteroidal anti-inflammatory drugs (Isenberg et al., 2007; Sparatore et al., 2009), L-cysteine activated arylthioamide H_2S donor molecules (Martelli et al., 2013), caged gem-dithiol derivatives that release H_2S upon light stimulation (Devarie-Baez et al., 2013) and molecules that release H_2S in aqueous systems. Many of these compounds, that are now available commercially, have been reviewed elsewhere (Kashfi & Olson, 2013; Song et al., 2014). More recently, additional H_2S donors have been introduced including poly(ethylene glycol)-ADT (PEG-ADT) (Hasegawa & van der Vlies, 2014), and *S*-aroylthiooximes (Foster, Powell, Radzinski, & Matson, 2014). In some cases, molecules which target delivery of H_2S to specific organelles within cells, notably mitochondria, have been reported (Le Trionnaire, Perry, Whatmore, Wood, & Whiteman, 2013; Le Trionnaire et al., 2014). The advantage of all of these new drugs (c.f. NaHS) is their potential at least to provide a means to manipulate endogenous H_2S levels within biological systems over a longer time course and within the range of concentrations likely to be achieved naturally. This approach will provide researchers with a wider range of "tools" to probe the biological significance of H_2S and, perhaps in due course, will provide clinicians with alternative therapeutics.

3. THE DEVELOPMENT AND CHARACTERIZATION OF GYY4137

GYY4137 (morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate) was described as a slow releasing H_2S donor with vasorelaxant activity both *in vitro* and *in vivo* in 2008 (Li et al., 2008). Interestingly, GYY4137 was initially developed as a chemical entity for use as an accelerator in the vulcanization of natural rubber back in the late 1950s but it was not until several decades later that its use as a potential drug candidate was envisaged. GYY4137 was the first organic small molecule, to be reported and characterized as a slow releasing H_2S donor (Fig. 1). GYY4137 is highly water soluble (>1 mg/mL at pH7.4), releases H_2S at a slow rate (unlike sulfide salts) and is relatively easy to synthesize chemically or can be obtained commercially. To date, the biological effects of

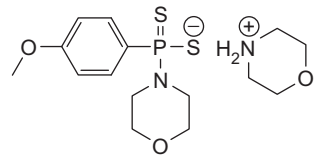

Figure 1 The chemical structure of GYY4137.

GYY4137 have been examined both *in vitro* and *in vivo* and a number of these studies will be described later in this article.

Initial work on the solution chemistry of GYY4137 indicated that it released low quantities of H_2S over a sustained period (hours to days) in aqueous solution (pH 7.4, 37 °C), (Li et al., 2008). Moreover, the release of H_2S from GYY4137 was pH and temperature dependent, with less H_2S released at 4 °C and greater release at pH 3.0. Confirmation of the H_2S releasing properties of GYY4137 within biological settings has also been demonstrated. Release of H_2S from GYY4137 has now being independently verified using a range of assay procedures including use of the fluorescent probe 2,6-dansyl azide and 5,5′-dithiobis(2-nitrobenzoic acid) (Qabazard et al., 2014) alongside more refined procedures such as the H_2S selective polarographic electrode (Kolluru, Shen, Bir, & Kevil, 2013) and detection of H_2S using fluorescent HPLC analysis of monobromobimane derivatives (Shen, Peter, Bir, Wang, & Kevil, 2012).

4. FACILE SYNTHESIS AND CHEMICAL CHARACTERIZATION OF GYY4137

GYY4137 can be conveniently prepared by ring opening of Lawesson's Reagent (0.8 g, 2 mmol) with morpholine (0.87 g, 1 mmol) in dichloromethane (20 ml) solvent at room temperature. Upon stirring for about 2 h, the product slowly precipitates from the reaction mixture forming an ionic salt. This salt is filtered, washed with dichloromethane (10 ml, twice) to good purity. The solid is dried under vacuum at room temperature avoiding heat. GYY4137 is highly water soluble and care must be taken to avoid any aqueous work-up or washing with polar solvents. Once the solid is isolated, it is characterized *via* spectroscopic methods. Nuclear Magnetic Resonance (NMR) in D_2O solvent confirms the identity and structure of GYY4137, (^{1}H NMR (400 MHz, D_2O) δ 8.02–7.84 (m, 2H), 7.05–6.94 (m, 2H), 3.93–3.84 (m, 4H), 3.81 (s, 3H), 3.71–3.54 (m, 4H), 3.23–3.21

(m, 4H), 2.86 (dd, $J = 9.6$, 5.5 Hz, 4H). ^{13}C NMR (101 MHz, D_2O) δ 161.03 (d, J (C–P) = 3.0 Hz), 132.48 (d, J (C–P) = 12.8 Hz), 131.43 (d, J (C–P) = 109.7 Hz), 113.56 (d, J (C–P) = 14.6 Hz), 66.66 (d, J (C–P) = 11.7 Hz), 63.64, 55.49, 44.72 (d, J (C–P) = 1.8 Hz), 43.19. ^{31}P NMR (162 MHz, D_2O) δ 89.00 (s)). In the infrared spectrum a stretch at 650 cm^{-1} can be seen for the P=S double bond.

GYY4137 should not be heated or exposed to moisture over time. It is recommended to store the solid material under nitrogen at −20 °C. For biological testing, a fresh solid sample should be weighed after allowing to warm to room temperature. For each assay, a solution in water or DMSO as required can be prepared and used freshly for the assay.

H_2S release from GYY4137 can conveniently be quantified using a dansyl azide probe *in vitro* (Wang, Peng, Ni, Dai, & Wang, 2014). The recommended assay uses 2, 6-dansyl azide with a specific procedure in aqueous acetonitrile to ensure a good signal:noise ratio. Upon release of H_2S from GYY4137 the sulfonyl azide group of the probe molecule rapidly reduces to a fluorescent primary sulfonamide product. Fluorescence accumulates over the time of the assay and can be measured using a plate reader at frequent time intervals up to days or even weeks. The limit of detection for this assay is around 1 μ*M* of released H_2S. Concentrations of GYY4137 above 100 μ*M*, preferably incubated at 37 °C, are required to obtain a clear response.

5. BIOLOGICAL EFFECTS OF GYY4137: AN OVERVIEW AND POTENTIAL ROLE IN DISEASE?

A plethora of publications are now available describing one or other of the biological effects of NaHS in cells, tissues, and organs in health as well as in a range of experimentally induced disease states in animals. Any description of this body of work is beyond the scope of the current review but will be found elsewhere in this volume. In general, there is much less information available about the biological activity of any of the new classes of slow releasing H_2S donors noted above and, indeed, for some of these compounds biological characterization is lacking altogether. At present, GYY4137 is without doubt the best studied of these H_2S donors in biological terms and it is the findings from these studies that will be described here with an overview of the major biological effects of GYY4137 provided in Table 1.

Table 1 Chronological summary of the major experimental evidence documenting the pharmacological effects of the novel H_2S releasing molecule, GYY4137

Experimental model	Reported effect	Outcome	Reference
Rat aortic rings and perfused rat kidney *in vitro* and in the anesthetized rat *in vivo*	Vasorelaxant	Slow relaxation of rat aortic rings and dilated the perfused rat renal vasculature by opening vascular smooth muscle K_{ATP} channels	Li et al. (2008)
Lipopolysaccharide (LPS; 4 mg/kg, i.v.) administered anesthetized rats	Anti-inflammatory	Inhibited LPS induced TNF-α production in rat blood. Decreased proinflammatory cytokines levels, TNF-α, IL-1β, IL-6, nitrite/nitrate, C-reactive protein, and L-selectin *in vivo*	Li, Salto-Tellez, Tan, Whiteman, and Moore (2009)
NO and H_2S cross talk within the heart of mice	Contractition	Heart contractility induced by GYY4137, in the presence of the NO donor molecule sodium nitroprusside	Yong et al. (2011)
Anticancer activity in a murine xenograph model, subcutaneous transplantation of either HL-60 or MV4–11 cells	Inhibition of tumor growth	Administered daily—reduced tumor volume in HL-60 and MV4–11 injected animals	Lee et al. (2011)
A mouse model of acute joint inflammation	Anti-inflammatory	GYY4137 reduced synovial fluid myeloperoxidase (MPO) and *N*-acetyl-β-D-glucosaminidase (NAG) activity and decreased TNF-α, IL-1β, IL-6, and IL-8 concentration with joints	Li et al. (2013) and Li, Yang, et al. (2013)
Platelet activation and microvascular thrombus formation	Antithrombotic activities	GYY4137 TRAP-induced adhesion molecule expression. Prolongation of venular thrombus formation and tail-vein bleeding time	Grambow et al. (2014)

High fat fed apolipoprotein E(-/-) mice	Antiatherosclerotic activity	Decreased aortic atherosclerotic plaque formation and partially restored aortic endothelium-dependent relaxation. Decreased ICAM-1, TNF-α, and IL-6 mRNA expression, and superoxide ($O_2^{\cdot}$) generation in aorta. Increased aortic eNOS, phosphorylation expression of PI3K, enhanced Akt Ser(473) phosphorylation, and downregulated the expression of LOX-1.	Liu et al. (2013)
High fat fed C57BL/6J mice	Prevention of insulin resistance	Decreased phosphorylation of perilipin 1 and hormone sensitive lipase	Geng et al. (2013)
In vitro cell culture models across a range of immortalized and nonimmortalized cell lines	Anticancer properties	Exposure to NaHS or GYY4137 decreased cell survival more effectively in cancer cells. GYY4137, significantly increased glycolysis leading to overproduction of lactate, decreased anion exchanger, and sodium/proton exchanger activity. Combined effects leading to increased metabolic acid production, defective pH regulation, and cell death.	Lee et al. (2014)

5.1. Cardiovascular system: Vascular smooth muscle and platelet function

One of the defining moments which spawned interest in the field of H_2S biology was the finding that this gaseous molecule dilates blood vessels *in vitro* and *in vivo* (Hosoki, Matsuki, & Kimura, 1997). Since this discovery a number of the newly introduced H_2S donor molecules, including GYY4137, have been found to exert similar vascular effects. The first report using GYY4137 identified a persistent and slowly developing relaxation of precontracted rat aortic rings which was antagonized by glibenclamide indicating that, like NaHS, this effect of GYY4137 is mediated *via* opening of vascular smooth muscle K_{ATP} channels (Li et al., 2008). In a separate study, GYY4137 also relaxed precontracted mouse aortic rings (Bucci et al., 2012). In addition, GYY4137 dilated the perfused preconstricted rat renal vasculature indicating that it acts not only on large capacitance vessels (e.g., aorta) but also on small resistance blood vessels which govern organ perfusion and blood pressure (Li et al., 2008). Finally, this report demonstrated that chronic treatment of rats with GYY4137 resulted in a lowering of blood pressure both in normotensive and, to a greater extent, in spontaneously hypertensive rats (Li et al., 2008). The effect of GYY4137 on cardiac motility has yet to be determined but one report did indicate that this H_2S donor (like NaHS) could reduce the negative inotropic effect of NO (Yong et al., 2011). It should be noted whilst chronic treatment with GYY4137, using a dose regimen which reduced blood pressure for a period of several days, caused no apparent deleterious side effects in this study, a full screen for possible toxic side effects of GYY4137 has yet to be reported.

Apart from its effect on vascular smooth muscle, GYY4137 also has additional actions within the cardiovascular system. These include antithrombotic activity *via* inhibition of thrombin receptor agonist peptide (TRAP)-induced adhesion molecule expression and associated platelet activation (Grambow et al., 2014) as well as antiatherosclerotic activity in high fat fed, apolipoprotein $E^{-/-}$ mice (Liu et al., 2013). The latter study also showed pronounced effects of GYY4137 to inhibit expression not only of iNOS but also of P-selectin, lectin-like ox-LDL receptor-1, phosphorylated IκBα, NF-κB, ICAM-1, VCAM-1, and a range of chemokines including CXCL2, CXCR4, CXCL10, and CCL17 indicative of an anti-inflammatory effect which will be discussed in more detail later in this review. GYY4137 has also recently been reported to augment NO-mediated human platelet aggregation in response to collagen and

thrombin although the underlying mechanism is not yet clear (Ilkan et al., 2013). Similarly, and equally as important, is a potential role for H_2S in regulating diabetic myocardial injury. In this context, high glucose concentrations promote cellular damage within cardiac tissues *via* the formation of advanced endpoint glycation products, increased reactive oxygen species (ROS) production and the generation of a proinflammatory environment. GYY4137 protects cardiac myoblast cells (H9c2) against high glucose-induced cytotoxicity by activation of the AMPK/mTOR signaling pathway. The activation of AMPK orchestrates the metabolic responses involved in the maintenance of appropriate energy levels needed for cell survival (Beauloye, Bertrand, Horman, & Hue, 2011; Wei, Hu, Zhuang, Liao, & Li, 2014). Such effects of GYY4137 may be related to a generalized action on cell metabolism which will be discussed below.

5.2. Effect of GYY4137 on nonvascular smooth muscle

A similar smooth muscle relaxant effect of GYY4137 has now been described in a range of tissues including human airway smooth muscle cells *via* opening of sarcolemma K_{ATP} channels (Fitzgerald et al., 2014), mouse intrapulmonary airways by an effect on intracellular calcium release (Castro-Piedras & Perez-Zoghbi, 2013), pig bladder neck tissues again by opening K_{ATP} channels (Fernandes et al., 2013), pregnant rat myometrial smooth muscle cells (Robinson & Wray, 2012) and in the bovine ciliary artery (Chitnis et al., 2013). Intriguingly, GYY4137 can occasionally, and in defined conditions, exert the opposite effect on blood vessels. Thus, in animals with experimentally induced endotoxic shock, GYY4137 reverses the hypotensive effect (i.e., increases blood pressure) due to *E coli* lipopolysaccharide (LPS) injection (Li et al., 2009). This likely reflects the anti-inflammatory effect of GYY4137 and, among other mechanisms, its ability to reduce the expression of inducible nitric oxide synthase (iNOS) which generates massive amounts of vasodilator nitric oxide (NO) in this condition.

5.3. Inflammation: Is GYY4137 pro- or anti-inflammatory?

GYY4137 exhibits anti-inflammatory properties in cultured cells such as macrophages *in vitro* and in a variety of animal models of inflammation *in vivo*. The anti-inflammatory properties ascribed to GYY4137 are typically concentration dependent with effects seen *in vitro* at concentrations ranging

from 0.05 to 5 m*M* and an effective dose administered intraperitoneally in mice of 50–100 mg/kg. In terms of inflammation, GYY4137 has been most extensively evaluated for its effect on cultured cells *in vitro*. In most cases, GYY4137 reduces the functionality of inflammatory cells by inhibiting NF-κB signaling in the target cells. Thus, GYY4137 pretreatment reduces the secretion of proinflammatory mediators like interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), NO, and prostaglandin E_2 (PGE_2) (Whiteman et al., 2010) in mouse macrophages stimulated with LPS as well as in IL-8-stimulated airway smooth muscle cells (Perry et al., 2011). In human synoviocytes and articular chondrocytes, GYY4137 decreased LPS-evoked production of nitrite (NO_2^-), PGE_2, TNF-α, and IL-6 (Li, Fox, et al., 2013). Similarly, in human chondrocytes isolated from osteoarthritis tissues, GYY4137 reduces NO, PGE_2, IL-6, and MMP-13 synthesis from cells stimulated with IL-1β (Fox et al., 2012). Stimulation of human chondrocytes isolated from osteoarthritis sufferers with IL-1β activates the NF-κB signaling pathway and the nuclear translocation and accumulation of this proinflammatory transcription factor. Induction of the NF-κB pathway is accompanied by increased production of the proinflammatory mediators PGE_2, IL-6, and expression of proinflammatory proteins like MMP-13, cyclooxygenase-2 (COX-2), and iNOS, (Burguera, Vela-Anero, Magalhães, Meijide-Faílde, & Blanco, 2014). In this study, both GYY4137 and NaHS significantly reduced the levels of proinflammatory molecules and proteins in chondrocytes. An inhibitory effect of GYY4137 on NF-κB signaling has also been described in mouse macrophages infected with *Mycoplasma fermentans*. In this study, GYY4137 reduced the production of the proinflammatory cytokine MCP-1 and diminished nuclear accumulation of the NF-κB heterodimer p65/p52 (Benedetti et al., 2014).

GYY4137 also exhibits anti-inflammatory activity in animals *in vivo*. For example, injection of GYY4137 prior to LPS administration in the rat protects against the ensuing endotoxic shock both in terms of partially restoring the depressed blood pressure and with respect to inhibiting the generation of proinflammatory cytokines and NO (Li et al., 2009; Wang, Liu, et al., 2014). In addition, GYY4137 administered 18 h after intra-articular injection of Complete Freund's Adjuvant (CFA) in the mouse reduced paw swelling but when administered 1 h before CFA, GYY4137 increased swelling suggesting that the effect of this donor in this animal model critically depends on the stage of the inflammation. This data implies that H_2S exerts different (perhaps even opposite) effects on the inflammatory response (Li, Fox, et al.,

2013; Li, Yang, Long, Yang, & Shen, 2013). In a recent report, GYY4137 coadministered with histamine into mouse skin inhibited the accompanying pruritis but did not affect the extravasations response (Rodrigues et al., 2013) again suggesting differences in the response to GYY4137 in different systems. Finally, administration of GYY4137 to newborn mouse pups subjected to hyperoxia (85% O_2 for 10 days) augmented lung alveoli development whilst blunting leuokocyte infiltration into the alveolar spaces indicative of an overall anti-inflammatory effect (Madurga et al., 2014). Cumulatively, these studies show that GYY4137 consistently reduces the expression and/or generation of proinflammatory mediators *in vitro* but has a more complex pattern of effects in animal models of inflammation *in vivo*. Inflammation is a multifactorial process which depends on the concerted and time-dependent interplay of numerous cell types each of which is able to generate a range of both pro- and anti-inflammatory mediators. Bearing in mind the somewhat promiscuous effect of H_2S on different cells and transduction mechanisms it is perhaps not surprising that the *in vivo* effects of GYY4137 are complex. It is clear from *in vitro,* and from some studies *in vivo,* that the anti-inflammatory effect of GYY4137 is mediated by inhibiting NF-κB signaling. However, this by no means rules out other possible molecular targets for GYY4137 derived H_2S which should continue to be sought to obtain a more complete picture of the mechanism of action of this H_2S donor.

5.4. Effect of GYY4137 in the reproductive system

The effect of GYY4137 has also been examined in the reproductive system. Roles for H_2S in female reproductive biology have only recently come to light. For example, both CBS and CSE are expressed in human intrauterine tissues and placenta (Patel, Vatish, Heptinstall, Wang, & Carson, 2009; You et al., 2011) as well as in epithelial cells of the fallopian tubes (Ning et al., 2014). The first direct evidence suggesting a role for H_2S in reproductive biology was in preeclampsia, a hypertensive condition that affects around 4% of all pregnancies. The administration of DL-propargylglycine (PAG, an inhibitor of the H_2S synthesizing enzyme, CSE) to pregnant mice resulted in hypertension and the promotion of abnormal vascularization within the placenta. This was correlated with reduced placental growth factor production and increased soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglinin (sEng) release from human umbilical vein endothelial cells which are considered to be markers for the onset of preeclampsia in

humans. In mice, GYY4137 reversed this effect of PAG treatment, reducing circulating sFlt-1 and sEng levels and restoring fetal growth (Wang et al., 2013).

In the human fallopian tube, epithelium cell-derived H_2S has been postulated to promote relaxation of the oviduct. Interestingly, egg retention within the fallopian tube is associated with ectopic pregnancy and deregulation of fallopian tube contractility may play a part in this process. Indeed, disruption of H_2S signaling has recently been reported to promote embryo retention and cause developmental delay in the mouse whilst pharmacological treatment with either GYY4137 or NaHS reversed these effects and restored the status quo (Ning et al., 2014).

Although more work is clearly needed, these limited data do suggest possible roles for H_2S in protecting against conditions such as preeclampsia and ectopic pregnancy and thereby raise the possibility that an H_2S donor, like GYY4137, may be of value therapeutically in this regard.

5.5. GYY4137: Apoptosis and cell cycle progression

Whilst most reports in the literature have concentrated on the effect of GYY4137 in the cardiovascular system and in inflammation, it is worth noting that a potential use of this drug in the treatment of cancer has also recently begun to be addressed. To date, only a few reports have examined the involvement of H_2S in cancer cell signaling and/or in the induction and promotion of apoptosis in cancer cells. From the available literature, a number of key findings have been made about the anticancer properties of H_2S and donor molecules. Importantly, both pro- and anti-apoptotic effects of H_2S have been reported perhaps related to the choice of H_2S generating system used. For example, as noted earlier, conventional H_2S donors such as NaHS and Na_2S generate H_2S rapidly in aqueous systems and the released H_2S has a very short half life in cell culture media. It might be argued that GYY4137, which releases H_2S slowly and continuously, may therefore be a better "tool" with which to evaluate the longer term effects of the gas in cell culture systems.

With this in mind, GYY4137 has been shown to influence cell cycle progression in a range of different cell types. This effect of GYY4137 appears to be cell type specific since it caused G2/M phase cell cycle arrest in immortalized MCF-7 cells (Lee et al., 2011), G1/S cell cycle transition in HepG2 cells (Lu, Gao, Huang, & Wang, 2014), and inhibits abnormal proliferation in lymphocytes derived from systemic lupus erythematosus

patients (Han et al., 2013). Aside from affecting cell cycle progression, GYY4137 also caused a concentration dependent loss of cell viability across a range of immortalized human cancer cell lines including HeLa, HCT-116, Hep G2, HL-60, MCF-7, MV4-11, and U2OS (Lee et al., 2011). Preliminary work has revealed that a number of GYY4137 chemical analogues exhibit similar activity (Liao et al., 2013). Mechanistic studies revealed that these effects were mediated *via* a caspase dependent pathway since PARP-1 and caspase 9 cleavage were evident in GYY4137-treated MCF-7 cells (Lee et al., 2011). The same study also reported that GYY4137 exerted a dose-dependent inhibitory effect on tumor growth in the mouse *in vivo*. Likewise, GYY4137 suppressed cell proliferation in human hepatocellular carcinoma (HCC) cell lines and reduced tumor growth *in vivo* most likely, in this case, by inhibition of the signal transducer and activator of transcription 3 (STAT3) pathway (Lu et al., 2014).

GYY4137 alters cell metabolism in a number of different ways some of which may have relevance in terms of its antitumor activity. For example, treatment of HeLa cells with GYY4137 increased calcium signaling by upregulating expression of the inositol 1,4,5-trisphosphate (IP3) receptors 1 and 2 (Lencesova et al., 2013). Moreover, depletion of calcium stores from the endoplasmic reticulum (ER) is accompanied by an increase in the expression of X-box, CHOP and ATF4, known biomarkers associated with ER stress (Lencesova et al., 2013). Similarly, upregulation of the sodium calcium exchanger (NCX) by GYY4137 elevates cAMP levels leading to increased expression of β_1 and β_3 adrenoceptors and the induction of apoptosis (Markova et al., 2013). These findings raise the possibility that GYY4137 may promote endoplasmic reticular stress *via* mobilization of calcium stores.

Historically, H_2S gained notoriety due to its ability to inhibit the catalytic activity of cytochrome C oxidase, as H_2S directly reduces the oxygen-reactive a3CuB binuclear center of this protein (Nicholls, Marshall, Cooper, & Wilson, 2013). It is perhaps no great surprise therefore that recent work has indicated subtle effects of GYY4137 on primary metabolic pathways within cancer cells. Aerobic glycolysis provides cancer cells with a growth or survival strategy as it provides valuable metabolic intermediates needed to support rapidly proliferating cells (Gatenby & Gillies, 2004). GYY4137 has recently been reported to inhibit glycolysis across a range of immortalized cancer cell lines with little or no effect on noncancer cells. Interestingly, overproduction of lactate and inhibition of the anion exchanger and sodium/proton exchanger lead to defective pH

regulation, increased intracellular acidification, and cell death (Lee et al., 2014).

Clearly more work is needed to determine the role of extrinsic and intrinsic signaling pathways in the apoptotic effects of GYY4137 and to assess in greater detail its antitumor action *in vivo*. Moreover, the precise molecular mechanisms observed in these experimental studies also need to be examined.

5.6. GYY4137 and aging

Aging is intrinsically linked to many of the pathophysiological diseases described in this review. Any description of the biology of the aging process is beyond the scope of this review but readers will undoubtedly be aware of the profound and often deleterious effects that the aging process has on the cardiovascular system, metabolic functions, and immune system of mammalian species. Recently, it has been hypothesized that H_2S plays a fundamental role in the aging process (Zhang et al., 2013). Evidence that H_2S plays a role in this process stems from a number of observations notably, that H_2S can inhibit oxidative stress (Benetti et al., 2013; Whiteman et al., 2004), increase longevity in some model organisms (Miller & Roth, 2007) and promote the induction of genes associated with longevity (Lee, Kennedy, Tolonen, & Ruvkun, 2003; Qabazard et al., 2014). H_2S also reduces cell senescence and promotes cellular cytoprotective systems (Predmore, Alendy, Ahmed, Leeuwenburgh, & Julian, 2010). Indeed, these studies provided a potential approach to treat age-related deterioration in health. The big question is how does H_2S influence the aging process and can GYY4137 be utilized as an antiaging drug in the sense of reducing the incidence or severity of age-related diseases? Recent evidence has indicated that GYY4137 can indeed reduce the deleterious effects of the aging process at least in the model nematode worm *Caenorhabditis elegans* (Qabazard et al., 2013, 2014). For example, GYY4137 treatment increased the expression of several age-related, stress response, and antioxidant genes. Moreover, GYY4137 increases lifespan in short-lived mev-1 mutants with elevated oxidative stress and protected wild-type *C. elegans* against paraquat induced poisoning which is believed to occur *via* the generation of an excess of superoxide anions (Qabazard et al., 2013, 2014).

Taken together the available literature points to a fundamental role for H_2S signaling in the aging process and provides rational avenues of intervention using H_2S donor molecules like GYY4137.

6. THE EFFECT OF GYY4137 IN NONMAMMALIAN SYSTEMS

Whilst the majority of work highlighting a therapeutic role for GYY4137 has been derived from studies in mammalian models of disease less has been reported about the use of GYY4137 in other biological systems. This may be important since it provides further experimental evidence for biological roles for H_2S and likely bolsters additional, albeit nonclinical, translational uses, for example to alleviate stress within agronomic crops.

To date, only a few reports have studied the effect of GYY4137 in nonmammalian species (for summary, see Table 2). For example, GYY4137 prevents Na^+ uptake in larval zebra fish, *Danio rerio* (Kumai, Porteus, Kwong, & Perry, 2014) and has antiaging properties when tested in the nematode worm, *C. elegans* (Qabazard et al., 2013, 2014). The effect of GYY4137 on plant metabolism has also been evaluated. Multiple reports over the years have shown that plants respond to H_2S in a biphasic manner with both positive and negative effects on growth observed. From an environmental context, exposure of plants to high levels of exogenous sources of H_2S gas can have phytotoxic effects with common symptoms associated with but, not restricted to, reduced oxygen production, reducing nutrient uptake, and the promotion of leaf damage (Thompson & Kats, 1978). In contrast, increased rates of photosynthesis, germination rates, and increased plant productivity upon exposure to H_2S have also been documented (Chen et al., 2011; Dooley, Nair, & Ward, 2013). Interestingly, H_2S production in plant tissues is likely derived from the sulfur assimilation pathways and L-cysteine desulfydrase (Ravilious & Jez, 2012; Romero et al., 2014; Takahashi, Kopriva, Giordano, Saito, & Hell, 2011). It is known that in the presence of high concentrations of sulfate (SO_4^{2-}), sulfur dioxide (SO_2) and/or L-cysteine, plant tissues can synthesize, and emit H_2S (Hällgren & Fredriksson, 1982; Sekiya, Schmidt, Wilson, & Filner, 1982; Wilson, Bressan, & Filner, 1978). Surprisingly, it was not until recently that a potential physiological role for H_2S in plants was considered. To date, the reported effects of H_2S on plants are wide ranging and this has led to many researchers postulating that H_2S may function as a potential plant signaling molecule. Sadly, descriptions of potential roles for H_2S releasing donor molecules in plants are rare. The use of H_2S donor molecules should be considered in plant studies since H_2S has the ability to promote plant growth and vigor, reduce the effect of biotic and abiotic stress and increase crop yields (Dooley et al., 2013; Thompson, Kats, & Lennox, 1979).

Table 2 Studies utilizing GYY4137 to demonstrate novel physiological roles for H_2S across a range of model species

Experimental model	Reported effect	Outcome	Reference
Arabidopsis thaliana ecotypes Landsberg erecta and Columbia ecotypes	Stomatal function	GYY4137 promotes stomatal opening in the light and prevented stomatal closure in the dark. Reduces the accumulation of NO within guard cells	Lisjak et al. (2010)
Capsicum anuum	Stomatal function	Reduced accumulation of NO induced by abscisic acid (ABA) treatment of leaf tissues. Promotes stomatal opening	Lisjak et al. (2011)
Zea mays L., Yedan no. 51	Prevention of heat damage	GYY4137 increased endogenous H_2S levels, promoted survival, and reduced electrolyte leakage in seedlings under heat stress	Li, Fox, et al. (2013) and Li, Yang, et al. (2013)
Capsicum anuum	Prevention of salt stress	Accumulation of proline induced by salt treatment	Lisjak et al. (2013)
Caenorhabditis elegans	Aging	GYY4137 increased the expression of several age-related, stress response, and antioxidant genes. Increased lifespan in short-lived mev-1 mutants with elevated oxidative stress. Protected wild-type *C. elegans* against paraquat poisoning	Qabazard et al. (2014)
Caenorhabditis elegans	Survival and H_2S homeostasis	GYY4137, extended median survival and increased tolerance toward oxidative and endoplasmic reticulum (ER) stress. Restored life span in cysl-2 mutant worms.	Qabazard et al. (2013)

Such studies highlighting a fundamental role of H_2S in the regulation of aging, cellular stress pathways, and in plant physiological responses.

GYY4137 has already been used to explore the role of H_2S in regulating stomatal function in plants (Lisjak et al., 2010, 2011). In these studies it was noted that H_2S, derived either from NaHS or GYY4137, caused stomatal opening in the model plant *Arabidopsis thaliana* and in the commercially important crop species, *Capsium anuum*. Further experimentation revealed that both donors reduced abscisic acid induced accumulation of NO within leaf tissues suggestive of a potential cross talk between the two gases. Similarly, García-Mata and Lamattina (2010) also reported that GYY4137 promotes stomatal closure in *Vicia faba* (*L.*) *var. major* and in *Impatiens walleriana*.

The involvement of H_2S, derived from GYY4137, in the prevention of plant stress responses has also recently been explored. In plants, exposure to high temperatures increases membrane fluidity and reduces membrane integrity, promotes protein denaturation, and reduces chloroplast and mitochondrial function leading to a state of oxidative and osmotic stress. In this regard, GYY4137 reduces the impact of heat stress and preserves the viability of *Zea Mays* (Li, Yang, et al., 2013). Moreover, preliminary data shows that GYY4137 induces expression of the cytoprotective amino acid proline in plant tissues following salt stress (Lisjak et al., 2013).

7. CONCLUSION

Much progress has been made in the characterization of the pharmacological effects of GYY4137 over the past several years. The biological activity of GYY4137 have now been comprehensively evaluated in a range of *in vitro* cell systems as well as in mammalian and nonmammalian species *in vivo* and also in plants. It is now clear that GYY4137 exhibits a very wide range of biological effects from vasodilator activity in isolated blood vessels through antiaging activity in *C. elegans* to crop preservation in plants. GYY4137 has the advantage that it is not only very water soluble but also easy to synthesize chemically as well as being commercially available. GYY4137 was an early addition to the family of H_2S donors and can therefore be considered as the prototype for this class of compounds. Further progress will undoubtedly lead to new second and third generation compounds with the ability to release their H_2S "payload" at well-defined rates, in defined organs and cells and in the appropriate subcellular location. Experimental "tools" of this type will prove invaluable in unraveling the complex biological roles of H_2S in multiple systems and perhaps also act as the forerunners of a new class of therapeutic agents.

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