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REVIEW

Pharmacological tools for hydrogen sulphide research: a brief, introductory guide for beginners

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The purpose of this brief review is to help researchers in their initial approach to the H_2S field and to provide answers for the most frequently posed questions by newcomers to the topic related to H_2S donors and inhibitors of H_2S synthesis, as well as methods to measure H_2S production. Here the reader will find a practical guide that provides fast and to the point information on how to (i) deliver H_2S to cells; (ii) modulate its endogenous production; and (iii) measure its levels in fluids, cells and tissues in order to gain an understanding of its role in health and disease.

LINKED ARTICLES

This article is part of a themed section on Pharmacology of the Gasotransmitters. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-6

Abbreviations

3-MST, 3-mercaptopyruvate sulphur transferase; 3MP, 3-mercaptopyruvate; ADT-OH, anethole dithiolethione-OH; AOAA, aminooxyacetic acid; AVG, aminoethoxyvinylglycine; BCA, β-cyano-L-alanine; CBS, cystathionine-β synthase; CSE, cystathionine-γ lyase; DADS, diallyldisulphide; DATS, diallyltrisulphide; GYY4137, 4-methoxyphenyl(morpholino) phosphinodithioate-morpholinium salt; PAG, propargylglycine; PLP, pyridoxal-5′-phosphate; TBZ, thiobenzamide

Table of Links

TARGETS	LIGANDS
H_2S	Cysteine
CAT	Homocysteine
CBS	AOAA
CSE	Propargylglycine
MPST	3-mercaptopyruvate

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Hydrogen sulphide (H_2S) has been proposed as the newest member of the gasotransmitter family that also includes •NO and carbon monoxide (CO). Until the early 1990s, publication of H_2S-related research was restricted to toxicology journals, reflecting the limited interest of the general bioscience community for this molecule. Currently, papers for H_2S are frequently published in many biology and medical journals that cover topics such as biochemistry, physiology, pharmacology and pathophysiology. H_2S acts as a signalling molecule in the CNS, regulates smooth muscle tone, cell metabolism and growth, apoptosis, and migration (Kimura, 2011; Li *et al.*, 2011; Wang, 2012). It has, thus, been implicated in a plethora of physiological and pathophysiological processes from angiogenesis and cancer to cardioprotection and atherosclerosis, as well as acute and chronic inflammation, thus attracting widespread attention among basic scientists and clinicians. Intense translational efforts to harness the therapeutic potential of H_2S are under way in academic and biotechnology laboratories.

The purpose of this ultra short review is to help researchers in their initial approach to the H_2S field. The most frequently posed questions by newcomers to the topic relate to the availability of H_2S donors and inhibitors of H_2S synthesis, as well as methods to measure H_2S production. We have put together a practical guide to provide fast and to the point answers on how to deliver H_2S, inhibit its endogenous production and measure this gasotransmitter. Several excellent comprehensive recent reviews on H_2S biology exist and the reader is referred to them for further reading. Although this report has been prepared by only three authors, the statements expressed herein reflect the consensus reached by the members of the European Network of Gasotransmiters (ENOG; http://www.gasotransmitters.eu) working on H_2S. The conclusions stated are the result of intense collaboration, vivid discussions and debates, and have emerged during the frequent meetings of the working groups within the network over the past two and a half years.

Endogenous production of H_2S

H_2S is generated both through enzymatic and non-enzymatic routes. H_2S can be generated via reduction of thiols and thiol-containing molecules, in a non-enzymatic manner. Enzymatically-generated H_2S is produced by three enzymes: cystathionine-β synthase (CBS; EC 4.2.1.22), cystathionine-γ lyase (CSE; 4.4.1.1) and 3-mercaptopyruvate sulphur transferase (3-MST also known as MPST; EC. 2.8.1.2) (Szabo, 2007; Singh and Banerjee, 2011; Wang, 2012). An initial difficulty in acquiring information about these enzymes is that they are known in the literature by alternative/additional names; the reader is advised to utilize enzyme commission number when in doubt. The three enzymes differ in their subcellular localization pattern, regulation, substrate utilization and co-factor requirement (Szabo, 2007; Kimura, 2011; Wang, 2012). CBS is a haem-containing, pyridoxal-5′-phosphate (PLP)-dependent enzyme mainly found in the cytosol (Meier *et al.*, 2001; Banerjee and Zou, 2005) that accumulates in the mitochondria during ischaemic/hypoxic conditions (Teng *et al.*, 2013). CSE is also a cytosolic enzyme that produces H_2S in a PLP-dependent manner; its translocation to mitochondria has also been observed under stress conditions (Fu *et al.*, 2012). CSE, and particularly, CBS, catalyse a multitude of H_2S-generating reactions using cysteine and homocysteine as substrates. Cysteine can be converted to 3MP by two enzymes that have been shown to be identical, for example, aspartate aminotransferase (AAT) or cysteine aminotransferase (also known as L-cysteine-2-oxoglutarate aminotransferase, CAT). 3MP in turn is converted to H_2S by 3-MST (Nagahara *et al.*, 2013). Although 3-MST activity is not directly dependent on PLP, this cofactor is required for the generation of 3MP, as it is needed for AAT activity. 3-MST bares a mitochondrial localization signal and can be found both in cytosolic extracts and mitochondrial fractions.

How to inhibit H_2S production or action

Several strategies can be employed to block the production or action of H_2S including the use of H_2S traps, pharmacological inhibitors of H_2S synthesis, molecular approaches to knockdown or reduce expression or genetically ablate H_2S-producing enzymes. siRNA are commonly used to knockdown CSE, CBS or 3-MST expression. Homozygous mutants completely lacking CBS suffer from severe growth retardation and the majority of them die within 5 weeks after birth (Watanabe *et al.*, 1995). Heterozygotes exhibit moderate levels of hyperhomocysteinaemia and are commercially available. Global CSE knockouts (KO) have been generated by two different groups; however, differences in their phenotypes in blood pressure have been noted (Yang *et al.*, 2008; Ishii *et al.*, 2010). To reconcile the differences observed, a floxed CSE KO has been generated within the ENOG consortium and is awaiting phenotyping and characterization. 3-MST KO mice have been recently described. These mice display anxiety-like behaviour and have altered neurotransmitter levels (Nagahara *et al.*, 2013).

H_2S can be removed from solutions and biological systems by adding chemicals that trap H_2S. Two agents that have been used for this purpose are haemoglobin and hydroxocobalamin. In addition, because of its high affinity for H_2S, Zn^{2+} can be used to bind and remove H_2S. Unfortunately, haemoglobin and hydroxocobalamin, will also bind and quench the biological activity of •NO, and Zn^{2+} has a variety of other pharmacological effects. Generating a selective scavenger/trap for H_2S in analogy to c-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt] that is used to scavenge NO would undoubtedly be a major advancement in the field.

At least three compounds can be used to selectively inhibit CSE over CBS or 3-MST: aminoethoxyvinylglycine (AVG); β-cyano-L-alanine (BCA) and propargylglycine (PAG), all of which are commercially available (Asimakopoulou *et al.*, 2013). BCA is a reversible CSE inhibitor, while PAG acts in an irreversible fashion (Whiteman *et al.*, 2011). It should be kept in mind that although the DL-PAG is often used, only the L-isoform inhibits CSE; D-PAG is metabolized and

contributes to the toxicity of the compound *in vivo*. Both PAG and BCA exhibit limited potency and membrane permeability and are used at high concentrations (mM) (Szabo, 2007). PAG will not inhibit recombinant CBS even if used at 10 mM, but BCA blocks CBS activity at concentrations above 1 mM (Asimakopoulou *et al.*, 2013). As the mechanism of BCA, PAG and AVG-induced inhibition involves PLP, it comes as no surprise that these inhibitors block other B6-dependent enzymes, such as aminotransferases (Szabo, 2007; Whiteman *et al.*, 2011). The complete selectivity profile of BCA, PAG and AVG for the panel of 140 known PLP-dependent enzymes (Di Salvo *et al.*, 2011) remains unclear. Aminooxyacetic acid (AOAA) was initially reported as a CBS-selective inhibitor, but was recently shown to also block CSE. In fact, AOAA is more potent at inhibiting CSE compared with CBS (Asimakopoulou *et al.*, 2013). AOAA could, thus, be useful as a dual CBS/CSE inhibitor. Like PAG and BCA, AOAA, too, interferes with the catalytic action of PLP-dependent enzymes and when used at mM concentrations, will have off-target effects. Hydroxylamine that has been used as an H_2S-synthesis inhibitor blocks both CBS and CSE and also readily generates •NO (Whiteman *et al.*, 2011). Thus, selective inhibition of CBS can only be achieved by siRNA or genetic methods. Because of the mitogenic action of CBS in cancer cells, approaches to discover novel, selective CBS inhibitors have recently been reported in the literature. Similar to CBS, no selective and potent 3-MST inhibitor is available to date. Researchers have used α-ketobutyrate, α-ketoglutarate and pyruvate to inhibit H_2S production from 3MP *in vitro* (Whiteman *et al.*, 2011). In summary, it is impossible to convincingly prove the involvement of CBS or 3-MST in biological responses with the sole use of pharmacological inhibitors, which are often employed at mM concentrations. We recommend, that even for CSE-mediated phenomena, both pharmacological and molecular/genetic approaches should be used in order to reach valid conclusions.

How to use H_2S donors

The available donors can be divided based on their chemical nature and rate of release as follows: inorganic versus organic compounds and fast- versus slow-releasing molecules.

Inorganic generators

Administration of H_2S as a gas-saturated solution or as a gas mixture at a given ppm presents some problems. Strict safety measures should be used when storing and handling a diffusible toxic compound, and most researchers are not comfortable delivering drugs in a gas form. For these reasons, only a very limited number of studies in the literature use this approach to administer H_2S. The most widely used inorganic compounds to deliver H_2S are NaSH, Na_2S and $Na_2S_2O_3$. Both have been used extensively *in vitro* and *in vivo* in the literature. The reader should be aware that these compounds are salts, and as such, when dissolved, they quickly dissociate leading to the immediate formation of H_2S in a pH-dependent manner. Therefore, they are classified as fast H_2S generators. H_2S is generated from these agents in a burst, which is obviously in sharp contrast to the way H_2S is endogenously produced. It is anticipated that at least the first few minutes, and depending on the concentration used, cells or tissues exposed to inorganic salts might be faced with concentrations of H_2S that are well within its toxic range.

Organic donors

The organic donors available can be divided according to their source of origin into naturally occurring and synthetics compounds. The organic donors described include several different types of chemical structures that do not allow a simple classification and it is beyond the purpose of the present review. However, the reader should be aware that, contrary to inorganic salts, in general, the organic donors release H_2S more slowly and the rate of H_2S liberated may differ substantially depending on whether it is done in the absence or presence of biological material. Some donors require the presence of a nucleophile to release H_2S and some others have been proposed to only yield H_2S in tissues or homogenates, suggesting that they act as pro-drugs requiring enzymatic biotransformation. However, it should be stressed that the kinetics and molecular mechanism of release from most of the organic donors has been poorly characterized.

Compounds of natural origin

Several H_2S sources of natural origin have been described including diallyltrisulphide (DATS), diallyldisulphide (DADS) allyl isothiocyanate, E-ajoene, sulphoraphane, erucin and iberin (Kashfi and Olson, 2013). However, full characterization of many of these sulphide-delivering agents is lacking. The most widely used naturally occurring H_2S donors are the garlic constituents DATS and DADS, which have been characterized both *in vitro* and *in vivo* (Benavides *et al.*, 2007).

Synthetic compounds

Three types of organic molecules capable of increasing H_2S levels have been reported to date: substrates of H_2S-generating enzymes, agents that liberate H_2S spontaneously or after bioactivation, and hybrid molecules that carry a H_2S-donating moiety (typically anethole dithiolethione-OH, ADT-OH or thiobenzamide, TBZ) conjugated to a known drug (Kashfi and Olson, 2013). ADT-OH and TBZ hydrids have been generated to produce compounds that exhibit an improved safety or efficacy profile and are obviously not ideal when one wishes to study the effects of H_2S in a biological system as they are 'bi-functional' molecules. Cysteine and its analogues or derivatives (including S-allyl cysteine, S-propargyl cysteine and N-acetyl cysteine) can be converted to H_2S by CSE/CBS, but their ability to generate H_2S obviously depends on the levels of endogenous H_2S-producing enzymes. Synthetic H_2S donors include 4-methoxyphenyl (morpholino) phosphinodithioate-morpholinium salt (GYY4137), thio-aminoacids (thioglycine and L-thiovaline), N-(benzoylthio) benzamides and more recently, dithioperoxyanhydrides (Kashfi and Olson, 2013). GYY4137 is by far the mostly widely used donor by the scientific community in experiments where a slow rate of H_2S production is desired (Li

et al., 2008). Within our consortium, agents that release H_2S in the mitochondria have been generated by coupling ADT-OH or TBZ to triphenylphosphonium. In terms of rate of release, thioaminacids generate H_2S faster than GYY4137 in buffered solutions (Bucci *et al.*, 2012; Zhou *et al.*, 2012). A potential problem faced by those wanting to experiment with H_2S donors is that only GYY4137 is commercially available and compounds have to be provided by the research groups that generated them.

In conclusion, it is intuitive that breakdown of an organic compound to yield H_2S strongly differs from the kinetics of H_2S produced by a salt. In contrast to the $^{\bullet}NO$ field, where several NONOates with a well-characterized mode of NO release and accurately calculated half-lives exist, the H_2S field suffers from lack of similar reagents. In spite of these difficulties, compounds with clearly different rates of H_2S release exist, which researchers can use to accommodate their individual needs. Another important aspect of sulphide donors is the fact that in many cases, it is not really clear whether sulphide or the sulphide-releasing functional group of the donor used is responsible for the biological action observed.

How to measure H_2S

Whether inhibiting H_2S biosynthesis or increasing H_2S levels by substrate supplementation or donor administration, it is crucial that the amount of H_2S is measured to validate the effectiveness of the experimental intervention. Several methods have been devised to measure H_2S including spectrophotometric, fluorimetric, chemiluminescent and chromatographic approaches, nanotube sensors, and polarographic and sulphide anion selective electrodes (for a review, see Whiteman *et al.*, 2011; Nagy *et al.*, 2013). It has also been proposed that H_2S levels can be evaluated by measuring H_2S metabolites (thiosulphate and sulphate) as surrogate markers, much like measuring nitrite/nitrate, which provides an estimate of $^{\bullet}NO$ levels. This approach to measuring H_2S has not been used to any significant extent as it awaits validation that H_2S metabolites can indeed accurately reflect H_2S levels. Moreover, although enzymatic activity assays for CSE and CBS have been reported, these are rarely used.

The three most important factors determining whether a method will be widely employed by researchers are ease of application, sensitivity and reproducibility. Methods requiring sophisticated, expensive and complex instrumentation or skills will be with treated with limited enthusiasm and will have difficulty becoming the method of choice. Most of the colorimetric and fluorometric methods currently used have a limit of detection of around 1 μM or higher and have, thus, difficulty detecting endogenously produced H_2S. In addition, many of the methods, although capable of accurately measuring H_2S generated by donors in aqueous buffers, cannot be used easily in biological samples because of the presence of cellular thiols and other substances that interfere with colour or fluorescence development.

In addition to free H_2S, acid-labile (in Fe–S complexes) and sulphane sulphur (e.g. thiosulphate, persulphides and polysuphides) are important pools regulating the bioavailable sulphur (Wang, 2012). Acid-labile sulphur and sulphane sulphur are readily measured in acid conditions and following reduction respectively (Ubuka, 2002). The most widely used method to determine H_2S is the methylene blue method (Stipanuk and Beck, 1982). It has the advantage of being easy to set up and use as it consists of a rather simple colorimetric procedure that requires determination of optical density in a spectrophotometer at the end of the procedure. Major drawbacks include a high limit of detection (>1 μM), interference with coloured substances and a non-linear behaviour of the calibration curve. Above all, the method measures not only H_2S, but also sulphide from iron–sulphur centres that are ubiquitous in biological systems, because of the acidity of the reactants used in the procedure. In spite of these problems it is preferred by most labs and relative differences in H_2S levels in control and treated samples can be easily picked up in many cases. It should be kept in mind that the absolute values often mean very little, and it is the relative difference between groups that confirm that the pharmacological treatment (e.g. CSE inhibition) together with other biochemical indices, has worked.

Gas chromatographic methods have been used mainly to determine H_2S as a pollutant. Conversely, HPLC methods have been used to determine H_2S in biological material, and they all include a derivatization step. At present, the most widely used derivatization method is the one employing monobromobimane (Wintner *et al.*, 2010). A simpler version of this method that uses dibromobimane is also available as fluorimetric method (Zhou *et al.*, 2012). In the past three years, a great number of fluorimetric probes have been reported by many different research groups (Lin and Chang, 2012). Members of our consortium have access to a great number of them and have tested these probes under different conditions. A common problem with the probes developed to date is their inability to measure endogenously produced H_2S in biological samples (cells, plasma, tissues, homogenates or cell culture supernatants). In summary, no ideal technique to measure H_2S exists, but available probes and methods, in spite of their limitations, can provide information about the efficacy of the pharmacological intervention.

Concluding remarks

Interest and knowledge regarding the importance of H_2S in mammalian systems has grown rapidly over the past 10 years. However, it is accepted by most of the investigators working in the field that this research area is still in its infancy. Although the situation has improved, better tools and reagents are urgently needed, and only a few have started to emerge. We hope that this short review will encourage and aid researchers who are considering joining H_2S research. This will help the field reach the critical mass needed in order to unravel the undoubtedly important biological properties and roles of H_2S and to explore the translational potential of H_2S.

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Author contributions

G. C. and A. P. had the idea of writing of this review and also wrote it. M. W. revised and collaborated for the chemical part.

Conflict of interest

Authors declare that they have no conflict of interest.

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