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The evolving landscape for cellular nitric oxide and hydrogen sulfide delivery systems: A new era of customized medications

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Abstract

Nitric oxide (NO) and hydrogen sulfide (H_2S) are industrial toxins or pollutants; however, both are produced endogenously and have important biological roles in most mammalian tissues. The recognition that these gasotransmitters have a role in physiological and pathophysiological processes has presented opportunities to harness their intracellular effects either through inhibition of their production; or more commonly, through inducing their levels and or delivering them by various modalities. In this review article, we have focused on an array of NO and H_2S donors, their hybrids with other established class of drugs, and the various engineered delivery platforms such a fibers, polymers, nanoparticles, hydrogels, and others. In each case, we have reviewed the rationale for their development.

Keywords

Nitric oxide; hydrogen sulfide; NO-releasing compounds; H2S-releasing hybrids; delivery platforms; fibers; polymers; nanoparticles; hydrogels

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1. Introduction

Nitric oxide (NO) is a ubiquitous gaseous free radical and hydrogen sulfide (H_2S) a gas that bears the pungent smell of rotten eggs; both are toxic, yet they are recognized as having multiple roles in normal physiology. In 1992, the journal *Science* referred to NO as the "Molecule of the Year" and in 1998 the Nobel Prize in Physiology and Medicine was awarded to Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad for the major discoveries surrounding it. Thus, for many years NO reined supreme as a signaling wonder molecule. However, in 1996 a new player came on the scene when Abe and Kimura in a landmark study established the physiological role of H_2S as a neuromodulator [1]. The elucidation of relevant enzymes and cellular signaling mechanisms led to the induction of H_2S into a family of small molecule signaling compounds called gasotransmitters. A term first used by Wang in 2002, gasotransmitter refers to the gaseous nature of these compounds at standard temperature and pressure [2]. There are currently three compounds that qualify as gasotransmitters: carbon monoxide (CO), nitric oxide (NO), and H_2S. For a molecule to be considered a gasotransmitter, three specific criteria must be met: 1) Endogenous production; 2) Free permeability through cell membranes; and 3) Well-defined biological targets and functions [2]. Over the years, much attention has focused on creating well-defined chemical tools to probe the NO and H_2S physiology in an attempt to determine their signaling roles in biological systems. The acknowledgement of NO and H_2S as gasotransmitters has led to an interest in pharmacological application of these gases. To that end, many NO and H_2S "releasing" compounds, also termed "donors", have been developed/designed that have been and are continuing to undergo intensive investigation. However, a limiting factor to the use of NO and H_2S as therapeutic agents are their delivery to the target organs, in many cases in a sustained manner. In this review we provide an overview of the various NO and H_2S-donating single agents and hybrid compounds together with the various platforms that are used for their delivery. This is an evolving landscape that is at the interface of basic life/physical sciences and complex medical applications.

2. Endogenous production of NO and H_2S

NO is synthesized in all cells by nitric oxide synthase (NOS)-dependent (L-arginine- NO pathway) and independent (nitrate-nitrite-NO pathway) pathways (Fig 1). NO is synthesized by three major isoforms of NOS including constitutive neuronal (nNOS/NOS1), endothelial (eNOS/NOS3), and inducible (iNOS/NOS2), reviewed in [3]. The expression levels of these enzymes varies in different tissues; nNOS and eNOS produce low concentrations of NO for short periods of time, whereas iNOS produces relatively higher levels and for longer time periods. NOS-independent NO production from nitrate and nitrite comes from the stomach following protonation of swallowed salivary nitrite (Fig 1) [4]. For details and in-depth presentation of this general area please see Gheibi et al in this special issue [5].

Endogenous production of H_2S is a result of direct enzymatic desulfhydration of cysteine, catalyzed by cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS), and indirect desulfhydration catalyzed by 3-mercapto-sulfurtransferase (3-MST) in the presence of reductants (Fig 2) [6]. CBS is present mostly in the central nervous system and the liver, while CSE is primarily responsible for H_2S production in the cardiovascular system. 3-MST

is located predominantly in the mitochondria and produces H_2S in concert with cysteine aminotransferase (CAT) [7–11]. Non-enzymatic production of H_2S (Fig 2) is responsible for a limited amount of H_2S in mammalian cells [12] and is mediated through reducing elemental sulfur or organic polysulfides via glucose-supported and thiol-dependent reactions [13–15].

3. NO and H_2S signaling, their interactions and cross talk

NO reacts with the active site of soluble guanylate cyclase (sGC) and produces cyclic GMP (cGMP) (Fig 3). cGMP activates cGMP-dependent Protein Kinase G (PKG), which phosphorylates multiple substrates [16]. In general, an increase in cGMP leads to smooth muscle relaxation, vasorelaxation, and decrease of platelet aggregation [17, 18]. NO can modify proteins by *S*-nitrosylation of cysteine residues [19–21], which may lead to either progression or inhibition of various diseases [22]. *S*-nitrosylation of NF-κB and matrix metalloproteinase 9 (MMP9) promotes cell death whereas *S*-nitrosylation of caspase-3, caspase-9, and c-Jun N-terminal kinase prevents activity and inhibits apoptosis [23]. Hypoxia-inducible factor-1 (HIF-1), estrogen receptor and NF-κB are redox sensitive transcription factors that are regulated by *S*-nitrosylation [24].

H_2S can also increase cGMP levels by inhibiting PDE5A, the enzyme that is involved in its catabolism [25]. H_2S interacts with ATP-sensitive potassium (K_{ATP}) channels leading to vasorelaxation in vascular smooth muscle [26] and enhancing cardiovascular function [27]. Voltage-dependent calcium channels are also important targets of H_2S [28]. H_2S signaling may be through sulfuration (*S*-sulfhydration) of target proteins, where a sulfhydryl group (-SH) is transferred to a cysteine residue to form hydropersulfide (-SSH) [29, 30] or persulfide groups [31]. H_2S also reacts with *S*-nitrosothiols to form thionitrous acid (HSNO), the smallest *S*-nitrosothiol [32]. HSNO can be metabolized to NO^+, NO, and NO^-, all of which have distinct physiological effects. Thus, HSNO can act a signaling molecule that may play a key role in cellular redox regulation [32].

NO and H_2S bind avidly to hemoglobin [2] leading to the formation of nitrosyl hemoglobin and sulfhemoglobin, respectively [33]. This competition for the common hemoglobin sink can potentiate the biological activity of the other. NO and H_2S can interact with each other, affecting each other's bioavailability and reactivity [34–36]. For example, NO inhibits CBS activity by binding to the heme group of the enzyme [37] and NaSH inhibits a recombinant form of bovine eNOS by an interaction between co-factors such as NADPH or tetrahydrobiopterin [38, 39]. NO can increase H_2S biosynthesis through increases in expression of CBS and CSE enzymes in vascular smooth cells [26, 40]. H_2S increases NO levels by increasing IL-1β-induced iNOS expression in vascular smooth cells [41] through NF-κB activation by a mechanism involving the ERK1/2 signaling cascade. In bovine arterial endothelial cells, H_2S has been shown to increase eNOS activation either indirectly by inducing its phosphorylation through an Akt-dependent mechanism [42] or directly by inducing Ca^{2+} release from the intracellular storage in the endoplasmic reticulum [43]. In human umbilical vein endothelial cells L-cysteine supplementation stimulated NO production, while inhibition of CSE blocked it [44]. Emerging data show that the interaction between H_2S and NO can also generate polysulfides (H_2S_n; H_2S_2 and H_2S_3) [45], reviewed

in [3]. Some of the main features of NO and H_2S signaling and their interactions are depicted in Fig 3.

4. Synthetic H_2S donors and H_2S-NSAID Conjugates

The need for chemical tools to study H_2S biology grew out of an interest in determining the physiological roles of H_2S. These tools include probes for the detection of H_2S, which change their spectroscopic properties in response to H_2S, CBS and CSE inhibitors, which reduce endogenous production of H_2S, and H_2S donors, which are compounds designed to release H_2S under specific conditions. To study H_2S in a physiologically relevant manner, donors with variable release rates and triggers are needed. We do not attempt to provide a comprehensive description of all available H_2S donors here; rather, we critically examine recent developments in H_2S donor chemistry, highlight specific examples of donors, and discuss important considerations in designing and choosing donors for biological studies and translation to H_2S-releasing therapeutics.

4.1. Small molecule H_2S donors

4.1.1. Sulfide salts—The most commonly used H_2S donors employed in biological studies are sodium hydrosulfide (NaSH) and sodium sulfide (Na_2S). Although routinely referred to as H_2S donors, sulfide salts are simply solid analogs of H_2S gas, providing instantaneous access to the biologically relevant forms of sulfide (H_2S and HS^-) in aqueous media. Sulfide salts have been extremely important in the establishment of H_2S as a gasotransmitter, and these salts have been applied to evaluate the biological roles and therapeutic potential of exogenous H_2S delivery.

One of the first studies on exogenous H_2S delivery by Wang used aqueous NaSH solutions in the in vitro evaluation of rat aortic rings [46]. Exogenously delivered NaSH led to a 60% greater relaxation versus controls, showcasing the properties of H_2S as a vasorelaxant. In a separate study by Du, exogenous delivery of NaSH via IV injection in rats with oleic acid-induced acute lung injury (ALI) alleviated symptoms by diminishing IL-6 and IL-8 levels, while simultaneously increasing IL-10 levels in the lung tissues [47]. This landmark study also provided verification of the hypothesis that down-regulation of endogenous H_2S production in the cardiovascular system is involved in ALI pathogenesis and disease progression.

Despite these successes in early work, Na_2S and NaHS are not ideal H_2S donors for several reasons. First, they are instantaneously converted into H_2S/HS^-, which is starkly different from biological H_2S signaling, which is tightly regulated by CSE and CBS. Second, commercial sulfide salts are impure, contaminated with polysulfides and other potential sulfur signaling species. Finally, without special precautions to prevent oxidation and volatilization, solutions of H_2S quickly become depleted, creating inconsistencies in the amount of H_2S added across a treatment group in an experiment because all animals or cell groups cannot be treated simultaneously.

4.1.2. Hydrolysis-Triggered H_2S Donors—Several types of H_2S donors have been developed that begin releasing H_2S as they dissolve in water. Release rates vary, and some

have pH-dependent release profiles. A few well studied classes of hydrolysis-triggered H_2S donors are shown in Figure 4.

4.1.2.1. Lawesson's Reagent and GYY4137: Lawesson's reagent (LR) is a chemical reagent used widely for the thionation of amides, esters, and ketones to their corresponding sulfur analogs [48]. LR is commercially available, making it a popular choice for biologists studying H_2S physiology. Importantly, LR releases H_2S in aqueous media over a much longer period than sulfide salts, making it a useful early candidate for the evaluation of sustained H_2S release. Much of the preliminary work in the field was performed with LR, but drawbacks, namely its low water solubility and lack of detailed kinetic analyses, led researchers to examine a water-soluble derivative of LR, called GYY4137. Synthesized via the reaction of morpholine and LR at room temperature, GYY4137 is easily prepared and purified before *in vitro* or *in vivo* administration. Many early studies on H_2S biology applied GYY4137 as an H_2S donor, but this compound also possesses drawbacks. Firstly, the preparation of GYY4137 yields the compound in a dichloromethane (DCM) complex, obfuscating biological data generated using the prodrug because DCM is metabolized into CO, another gasotransmitter with effects related to H_2S [49]. Also, a lack of proper control compounds in many studies using GYY4137 further complicates the interpretation of the observed biological effects with exogenous GYY4137 delivery.

Xian et al. more recently developed a series of donors with structures related to GYY4137 in the form of phosphonamidothioates, denoted as JK donors [50]. The synthesis of these compounds was accomplished by combining Lawesson's reagent with various amino acids, yielding a series of phosphonamidothioates analogous in structure to GYY4137. In their initial report, the authors found that in aqueous media at neutral and mildly basic pH, JK donors released low concentrations of H_2S. In contrast, under mildly acidic conditions ($pH \leq 6.0$), JK donors cyclized via nucleophilic addition of the carboxylic acid functionality of the amino acid, promoting H_2S release by breaking the weak P–S bond. Across the series of JK donors, lower pH accelerated release rates, while a GYY4137 control showed no release profile variability at various pHs. H_2S release profiles could also be tuned by altering the canonical R group substituent of the amino acid component of the donor. The authors observed that any substitution at the amino acid R group (i.e., $R \neq H$) promoted cyclization, and thus showed faster release profiles at neutral and basic pH over the unsubstituted donor. The JK donors (25 and 50 μM) showed efficacy in reducing cellular damage resulting from anoxia/reoxygenation (A/R) treatment with H_2O_2 in vitro. JK-type donors were also successful in reducing infarct size per area-at-risk via intracardiac injection in mice in an I/R model. JK donors, specifically JK-1, have been used in several biological studies since the initial report. In one key example, Lefer showed that JK-1 exhibited protective effects in multiple organs by reducing oxidative stress, improving exercise capacity, and attenuating rene-angiotensin-aldosterone system activation [51]. Due to their modular nature and activity, we expect JK-type H_2S donors to be a valuable chemical tool moving forward for investigating the cardioprotective effects of H_2S.

4.1.2.2. Dithiolthiones: 1,2-Dithiole-3-thiones (DTTs) are a group of compounds in the family of hydrolysis-triggered H_2S donors. There is also evidence that they are triggered by

intracellular enzymes, although the specific enzymes have not been identified [52]. Synthesized by the reaction of elemental sulfur and anethole, DTTs are easy to derivatize and can be readily attached to other molecules to make a wide variety of drugs and/or polymer-DTT conjugates. Substituted DTTs appear to hydrolyze cleanly, with the thione species being converted into a carbonyl [53]. However, in this study, complete hydrolysis required 48 h at 120 °C in a DMSO/H_2O mixture. The authors noted that hydrolysis under physiological conditions was very slow and did not present any data at 37 °C. However, they did observe activity of several DTTs as COX-1 and COX-2 inhibitors, with less potency noted for the hydrolyzed DTTs against both targets. Therefore, DTTs may have bioactivity aside from their potential H_2S-donating properties.

4.1.3. Thiol-Triggered H_2S Donors—Thiol-triggered H_2S donors are the most common class of non-hydrolysis-triggered synthetic donors. Free thiols are abundant nucleophiles in mammals and offer a platform from which thioldisulfide exchange can be used to accomplish H_2S release after nucleophilic attack. A number of thiol-triggered H_2S donors are shown in Figure 5.

4.1.3.1. *N*-Benzoylthiobenzamides: Among the first nucleophile-triggered H_2S donors were the *N*-(benzoylthio)benzamides developed by Xian and coworkers [54]. Synthesized from substituted derivatives of thiobenzoic acid, a series of *N*-benzoylthiobenzamides were assessed for H_2S release, with a range of different release rates observed. The H_2S release mechanism was confirmed with the formation of *N*-acetylcysteine, cystine, and benzamide. In cell studies, a selected *N*-(benzoylthio)benzamide protected human keratinocytes against methylglyoxal (MGO)-induced cell damage, an issue prevalent in diabetics [55]. These donors have shown cardioprotective effects in animal models of myocardial I/R injury, displaying a reduction in infarct size over controls [56].

4.1.3.2. Arylthioamides: Arylthioamides (ArC(S)–NH_2) are a class of donors that was first reported by Calderone and coworkers [57]. In this work, twelve arylthioamides were synthesized and evaluated for H_2S release. All donors released H_2S in response to cysteine. Release studies were conducted at relatively high concentrations of donor and thiol (1 mM and 4 mM, respectively), leading to rapid peak release time. The arylthioamides released only small amounts of H_2S, exhibiting maximum concentrations between 3–21 μM from 1 mM donor concentrations. The fast rise to a steady state concentration suggests these donors are fast-releasing compounds; however, this quick rise to maximum concentration in solution is quite misleading as the peak H_2S concentration represents a small fraction of the total available H_2S. Some of the donors released H_2S in the absence of a thiol trigger, indicating that they are not exclusively thiol-responsive. Alterations in ring electronics modulated release rates, but not in any clear pattern. One donor, *p*-hydroxybenzothioamide, was evaluated in a rat aortic ring contraction study and promoted vasodilation at 1 mM in the presence of noradrenaline (NA) without adding exogenous cysteine. Due to the sustained H_2S release profile of *p*-hydroxybenzothioamide and ease of conjugation to other compounds, arylthioamides have been conjugated to a variety of drugs as conjugates, including NSAIDs. In one key example, the development of a naproxen-hydroxybenzothioamide conjugate, ATB-346, was described [58]. The efficacy of ATB-346

as an anticancer drug was investigated, revealing that it induced apoptosis in human melanoma cells in animal studies. ATB-346 has also shown efficacy in reducing gastrointestinal tract injury while maintaining chemopreventative activity against colorectal cancer when compared to naproxen controls [59]. Further studies on ATB-346 are underway by Antibe Therapeutics, where a phase II GI safety study was completed in 2017.

4.1.3.3. *S*-Aroylthiooximes: *S*-Aroylthiooximes (SATOs) are a class of thiol-triggered donors developed by Matson and coworkers [60]. SATOs ($ArC(O)–S–N=CR_2$) are synthesized by condensation of an aryl aldehyde or ketone and an *S*-aroylthiohydroxylamine (SATHA, $ArC(O)–S–NH_2$) in the presence of catalytic trifluoroacetic acid in a reaction analogous to oxime formation. A series of substituted small molecule SATOs were synthesized by varying both the substituent on the SATHA and the aldehyde or ketone. SATOs released H_2S in the presence of cysteine and other thiols but did not show release in the presence of amines or water alone, suggesting SATOs possess stability in aqueous media. H_2S release was measured with the methylene blue method as well as amperometrically using an H_2S-sensitive electrode. A predictable electronics trend correlating the substituent on the SATHA ring with H_2S release was observed by fitting release half-lives to a Hammett plot. Under the conditions tested, H_2S release half-lives ranged from minutes to hours.

Although thiol-triggered H_2S donors have shown efficacy in releasing H_2S with well-defined release mechanisms, this subset of donors exhibits poor tissue targeting capabilities due to the ubiquitous nature of thiols inside cells. The design of thiol-triggered H_2S donors in the future must incorporate targeting capabilities to mitigate potential off-target effects. Strategies researchers could use to accomplish this important task include the use of targeting peptides, macromolecular scaffolds for increased circulation, and innovative molecular design with the capability for selective thiol reactivity.

4.1.4. Light- and Enzyme-Triggered H_2S Donors—Light-triggered prodrugs are useful tools for studies in vitro and hold promise as potential therapeutics due to the bioorthogonality of visible light as a trigger. Visible light possesses a unique advantage over other triggers because it enacts H_2S release without major perturbation of native biochemical processes. However, light application only triggers H_2S release in areas of the body where sufficient light penetration is possible. After prodrug administration, light of a particular wavelength can trigger release at the site of interest, potentially minimizing any off-target effects through direct spatiotemporal control over release.

Enzymes are of great importance to all living organisms and act on one or more specific biological substrates. In addition, utilizing enzymes as prodrug triggers often allows for specific targeting capabilities to a tissue of interest. Importantly, overexpression of enzymes is commonly symptomatic of many diseases, offering another layer of targeting capability to treat diseases with clever implementation of enzyme-triggered prodrugs. Several classes of light-triggered and enzyme-triggered H_2S donors are shown in Figure 6.

4.1.4.1. Geminal-dithiols: Xian reported one of the first examples of light-triggered H_2S donors in the form of geminal dithiols ($ArCH_2–S–C(CH_3)_2–S–CH_2Ar$).[61] The *ortho*-

positioned nitro group photolyzed when irradiated with UV light (365 nm) to produce an unstable geminal dithiol intermediate. This intermediate hydrolyzed to yield H_2S rapidly in aqueous media along with a benzyl alcohol byproduct. Xian's donors released their full payload within ~30 min, with no H_2S release being observed in the absence of UV light. Due to the acid-catalyzed hydrolysis mechanisms of gemdithiols, H_2S release was accelerated at low pH as compared to slower release at higher pH. Modifications of the bridging groups in some of the compounds yielded trends in release rate, with bulky aromatic bridging groups leading to slower release compared to faster release from alkyl bridging groups. These differences in observed release rates were the result of alterations in sterics and electronics.

4.1.4.2. α-Thioetherketones: Connal and coworkers developed a small molecule prodrug that incorporated a UV-responsive α-thioetherketone linkage with the ability to decompose into a thioaldehyde species and benzophenone, byproducts that are recognized as safe by the FDA [62]. The thioaldehyde generated H_2S in the presence of an amine, yielding an imine byproduct. Possessing similar functionality to Connal's thioetherketones, Singh and coworkers developed light-activated H_2S donors using a *p*-hydroxyphenacyl phototrigger [63]. The methylene blue assay validated efficient H_2S release from this family of donors with a maximum peaking concentration of 40 μM from 50 μM donor. Confocal microscopy confirmed Singh's donors released both H_2S and two equivalents of fluorophore in response to UV light (410 nm), providing a means of tracking H_2S release in real time. More recently, the authors reported on a water-soluble derivative of this light-triggered donor which reached peak H_2S concentrations of 45 μM in 30 min from 100 μM donor concentration, as measured by the methylene blue assay. Additionally, these donors exhibited no cytotoxicity towards HeLa cells at concentrations up to 20 μM both before and after photolysis [64]. This work exemplifies a step towards spatiotemporally controlled H_2S release.

4.1.4.3 Enzyme-Triggered H_2S Donors: Wang developed the first series of esterase-triggered H_2S donors [65]. The release mechanism of these donors hinges upon a well-known lactonization reaction named "trimethyl lock" (TML), which has been widely used to release a variety of drugs [66]. Wang's TML system first relies on the cleavage of a phenolic ester by an esterase, after which steric repulsion of three methyl groups places the resulting phenol in close proximity to a thioester, promoting lactonization. This cyclization reaction results in release of H_2S from the thiocarbonyl group. The authors synthesized several TML derivatives in this study through variations of the phenolic ester moiety and addition or removal of the methyl substituents on the aromatic ring. Because three specifically placed methyl groups are required to drive lactonization after ester cleavage, Wang proposed that removing these substituents would offer a means of slowing H_2S release in this system. As hypothesized, derivatives lacking aryl methyl groups exhibited longer release times, ranging from 45–99 min, while prodrugs containing the three methyl groups showed release rates ranging from 13–29 min. A variety of NSAID-TML hybrids were additionally synthesized and evaluated for their efficacy as anti-inflammatory agents, successfully inhibiting secretion of TNF-α.

In another example of enzyme-triggered H_2S donors, Chakrapani and coworkers utilized a protected geminal dithiol as an H_2S releasing moiety [67]. Instead of employing Xian's photocleavable functionality, the authors utilized a *para*-nitro benzyl thioether as a protecting group for the geminal dithiols. The nitro group on the benzyl linker underwent reduction to an amine in the presence of *E. coli* nitroreductase (NR). The resulting unstable aniline then underwent 1,6-elimination to release the deprotected geminal dithiol, which in turn rapidly hydrolyzed to generate H_2S and *p*-aminobenzyl alcohol as a byproduct. The donors showed H_2S release out to 45 min using a fluorescent BODIPY probe, with peak instantaneous H_2S concentrations reaching 30 μM in the presence of NR. In vitro studies using *E. coli* strains showed that the donor rescued the bacteria from oxidative stress caused by administration of common antibiotics, suggesting that H_2S production in bacteria may possibly be a mechanism leading to antibiotic resistance. Not much is known about the interactions of H_2S in prokaryote organisms, or about the function of H_2S in related areas of human physiology such as the microbiome. To elucidate the role of H_2S in these symbiotic systems and/or directly in prokaryotes, new varieties of chemical tools will need to be designed, synthesized, and tested.

5. Engineered H_2S delivery platforms

While small molecule donors comprise the vast majority of H_2S donors reported thus far, they possess several limitations when considering their use in biological systems. For example, many small molecule H_2S donors are inherently hydrophobic, which limits solubility in aqueous environments and may result in low bioavailability. Additionally, the reactive nature of H_2S donors incurs low stability in biological environments. Macromolecular donor systems offer a means to modulate the chemical, physical, and pharmacokinetic properties of a donor molecule without extensively changing its chemical nature. For example, hydrophobic donor molecules may be incorporated into hydrophilic polymers, either through covalent linkages or non-covalent sequestration, to improve solubility and circulation time. Importantly, the larger size of polymeric prodrugs allows for increased permeability in the leaky vasculature of tumors, providing additional targeting capabilities that small molecule donors usually do not possess [68]. Furthermore, the surrounding polymer structure may shield the incorporated donor motif against unintended degradation by biological nucleophiles. In addition, other materials such as peptides, nanoparticles, and inorganic assemblies may be used to encapsulate, bind, or covalently attach H_2S donors, providing modular release platforms with multiple tuning handles. As such, there has been increased interest in macromolecular H_2S donor systems; recent reviews provide an in-depth look at their development [69–71]. Here we provide a brief overview of some notable macromolecular H_2S donor systems and their contributions as biological tools for delivering H_2S. Of course, delivery of NO has many of the same challenges as H_2S delivery, and similar engineered systems have been developed for NO delivery, several of which are highlighted graphically in Figure 7. NO delivery systems are discussed in section 7.

5.1. H_2S-releasing polymers

5.1.1 ADT-Polymer conjugates—Perhaps the simplest strategy for creating macromolecular H_2S donor systems is the covalent attachment of donor molecules to linear polymers. To this end, a variety of covalently attached macromolecular H_2S donors have been developed. 5-(4-Hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (ADT-OH) is a popular DTT that can be easily appended to polymers via common coupling chemistry. ADT-OH is the active metabolite in anethole trithione (a drug used in the treatment of dry mouth) and possesses bioactivity aside from releasing H_2S, as noted above [72]. In addition, it lacks a clear mechanism of release, and the factors that affect release rate are not known. Despite these issues, ADT-OH has been conjugated onto several polymer systems, the first of which was reported by Hasegawa in 2014 [52]. In this work, ADT-OH was conjugated to the chain end of poly(ethylene glycol) (PEG), resulting in PEG-ADT. Conjugation to the polymer enhanced water solubility of the drug, significantly reducing toxicity *in vitro* in RAW-Blue macrophages. The PEG-ADT conjugates entered RAW cells through endocytosis, while small molecule ADT-OH predominantly diffused across the cell membrane. The authors attributed differences in cytotoxicity between small molecule and polymeric donors to a variance in intracellular distribution due to unique pathways of cellular entry. Furthermore, the slower release of H_2S from PEG-ADT relative to ADT-OH resulted in enhanced potentiating effects on LPS-induced inflammation in RAW-blue macrophages. The same group also created polymer micelles containing ADT units [73]. Similar to PEG-ADT linear polymers, the micelles showed less cytotoxicity than small molecule ADT-OH in vitro. Unexpectedly, PEG-*b*-PADT micelles enhanced the inflammatory response in gardiquimode-stimulated murine macrophages, whereas ADT-OH slightly decreased the inflammatory response under similar conditions. These studies collectively show the ability of polymeric and micellar systems to attenuate certain toxic effects of H_2S donors by controlling their entry into cells.

5.1.2. SATO-Polymer conjugates—In another example of macromolecular systems with covalently attached H_2S donor molecules, Matson and coworkers developed water-soluble polymers conjugated with H_2S releasing-SATO groups through a post-polymerization modification of pendant aldehydes [74]. In later work, the authors prepared amphiphilic block copolymers with SATO-functionalized hydrophobic segments, which readily formed spherical micelles in aqueous solutions [75]. Unlike small molecule and water-soluble polymeric SATO donors, H_2S release from SATO-containing micelles was limited by diffusion of triggering cysteine molecules into the hydrophobic micelle core. As a result, SATO-containing micelles experienced drastically slower H_2S release kinetics, exhibiting a 9-fold increase in release half-life relative to the small molecule SATO analog. Additionally, the polymer micelles showed greater efficacy in decreasing the viability of HCT116 colon carcinoma cells compared to small molecule H_2S donors, highlighting the significance of H_2S release rates in biological systems. To further expand this study, the authors developed a method for systematically tuning the H_2S release rate from SATO-containing micelles through control of micelle core mobility [76]. In this work, a plasticizing comonomer was incorporated into the core-forming block of SATO-conjugated polymer amphiphiles to produce a series of SATO-containing micelles with varying amounts of micelle core mobility. The H_2S release rate varied over 20-fold throughout the series of

polymer micelles, signifying that diffusion of triggering cysteine molecules into the micelle core could be precisely controlled by tuning the chemical composition of the core-forming block. Altogether, these studies show the potential for macromolecular H_2S donors in overcoming the challenges small molecule donors face in biological systems.

5.1.3. Arylthioamide-Polymer conjugates—In 2016, Davis et al developed amphiphilic block copolymers conjugated with H_2S-releasing arylthiobenzamide groups through thionation of pendant benzonitrile groups [77]. The authors controlled the placement of thiobenzamide groups to form amphiphilic block copolymers with H_2S-releasing groups in either the corona-forming or core-forming blocks. The polymer amphiphiles both formed micelles in aqueous buffered solutions. Faster H_2S release from corona-functionalized versus core-functionalized micelles was attributed to the shielding effect of the micelle core limiting the rate of hydrolysis for thiobenzamide groups sequestered within it. Furthermore, H_2S delivered from slow-releasing, core-functionalized polymer micelles produced a slow, sustained increase in cytosolic ERK signaling activity and a smaller but more rapid increase in plasma membrane-localized protein kinase C activity in HEK293 cells. These results demonstrate the potential for modifying specific cellular signaling pathways through release of H_2S with spatiotemporal control.

5.1.4. Geminal Dithiol-Polymer conjugates—As mentioned above, light-triggered donors are promising tools for spatiotemporally controlled release of H_2S in biological systems. As such, geminal dithiol donors were recently incorporated into macromolecular donor systems. Li reported a polymeric H_2S donor system based on conjugation of 2-nitrobenzenemethanethiol to pendant ketones on a water-soluble polymethacrylate [78]. The rate of H_2S release from the copolymer exhibited positive correlation with the UV light intensity, while in the absence of irradiation, no release was observed. Additionally, the water-soluble polymeric donor and its H_2S-releasing photodegradation product exhibited no cytotoxicity towards human fibroblast cells at concentrations up to 1 mg/mL. While this work lacked a demonstration of light-triggered release at a site of interest in a biological environment, it represents a promising step forward in the development of H_2S donors with spatiotemporally controlled release.

5.2. Microparticles and fibers

Macromolecular donors based on polymer assemblies (e.g. micelles or liposomes) and water-soluble polymers can circulate throughout the bloodstream, thereby delivering H_2S systemically. In contrast, larger polymer assemblies or supramolecular structures (e.g. microparticles or hydrogels) persist in the area where they are implanted, leading to a localized release of H_2S. Localized delivery can be particularly advantageous if the donor is implanted at a site of interest. Additionally, the larger size of these assemblies provides an increased shielding effect for donor moieties sequestered within them, leading to longer or sustained release of H_2S.

5.2.1. Microparticles—In 2015, Bowden et al. reported polylactide microparticles system functionalized with thiobenzamide groups as a system for sustained delivery of H_2S [79]. Ring-opening copolymerization of L-lactide and a 4-hydroxythiobenzamide-

functionalized lactide monomer afforded polymers decorated with pendant thiobenzamide groups along the backbone. From the functionalized polylactides, two sets of spherical microparticles were generated with average diameters of 12 ± 4 and 0.5 ± 0.1 μm. The microparticles experienced 10% weight loss after four weeks at pH 7.4, suggesting the potential for prolonged H_2S delivery. Degradation of the microparticles should result in increased exposure of H_2S-releasing thiobenzamide groups, therefore a long timescale of degradation should elicit sustained delivery of H_2S. However, the authors could not quantitatively measure H_2S levels due to low thiobenzamide loadings in the microparticles, slow microparticle degradation, and rapid loss of H_2S from aqueous solutions. Despite this limitation, this work demonstrates the potential for microparticle systems that can deliver H_2S in a sustained manner.

5.2.2. Electrospun Fibers—Wang and co-workers reported the first electrospun H_2S-releasing microfibers in 2015 based on a biodegradable polycaprolactone (PCL) polymer matrix.[80] A solution of PCL at different concentrations (6%, 8%, and 12%) and a thiol activated H_2S-donor (NSHD1) were subjected to electrospinning to yield microfibers with diameters ranging from 0.5 to 1.5 μm. An increase in microfiber diameter was observed with increasing PCL concentration. H_2S release half-lives for the microfibers were longer than NSHD-1 alone, with measurable H_2S levels extending past 24 h. Release rate depended on fiber thickness, with thicker fibers releasing more slowly than thinner ones. Additionally, the H_2S-releasing microfibers protected H9C2 cardiomyocytes subjected to oxidative stress by addition of H_2O_2. They also enhanced proliferation of 3T3 fibroblasts, which is potentially useful for wound healing. In later work, the authors incorporated JK-1, a hydrolysis-triggered H2S donor, into PCL fibers using a similar electrospinning technique [81]. The JK-1-doped PCL fibers showed an extended H_2S release profile over the small molecule in solution, which is expected for a hydrolysis-triggered donor. Furthermore, one-time application of PCL-JK1 nanofibrous scaffolds to full-thickness cutaneous wound models in mice showed successful wound regeneration over 20 d with increased healing rates relative to control non-doped PCL fibers.

5.3 Hydrogels

Beyond use in PCL microfibers, the JK-1 H_2S donor has also been encapsulated in a hydrogel system for the potential treatment of indivertible disc degeneration (IDD) [82]. Hydrogels are networks of polymer chains, held together by chemical or physical crosslinks, and expanded by water. In this work, JK1 was encapsulated within the porous network of a collagen hydrogel to generate Col-JK1 gel. The gel could be slowly degraded by MMP9, an enzyme that is overexpressed under IDD conditions. Degradation of the collagen gel caused release of JK1 molecules into the low pH environment of the inflamed tissue, which subsequently released H_2S. As expected, the shielding effect of the surrounding hydrogel structure resulted in slower H_2S release from Col-JK1 relative to the small molecule alone. Additionally, the presence of MMP9 led to increased degradation of the hydrogel structure, and accelerated release kinetics. Furthermore, Col-JK1 successfully inhibited apoptosis in nucleus pulposus cells and prevented degradation of extracellular matrix (ECM), indicating its potential for IDD treatment. While this system marks progress towards enzyme-triggered

macromolecular donors, substantial release of H_2S in the absence of MMP9 denotes the need for further development of these systems.

The robust SATO-forming reaction has also been leveraged to prepare H_2S releasing amphiphilic peptide systems, some of which form hydrogels. For example, an amphiphilic peptide with the sequence IAVEEE was modified by attaching an aryl aldehyde to the N-terminus to form a SATO-based aromatic peptide amphiphile [83]. The SATO-containing peptide amphiphiles self-assembled in aqueous media to form nanofibers that gelled in the presence of calcium, affording hydrogels using 1 wt.% peptide, which exhibited sustained H_2S release with a peaking time of ~120 min. In vitro studies using mouse brain endothelial cells showed minimal toxicity of the gels. More recently, a similar SATO-based aromatic peptide amphiphile system was testing for the treating occlusive diseases such as intimal hyperplasia (IH) [84]. The peptide gels inhibited vascular smooth muscle cell (VSMC) proliferation and IH in ex vivo human vein cultures. The peptide gels promoted HUVEC proliferation and transmigration, suggesting H_2S donor gels such as these could aid in recovery after vascular intervention. Other peptide-based H_2S-releasing SATO systems have also been recently reported [85–89]. We envision that SATO-peptide gels have a promising future for in vivo studies where localized H_2S delivery is imperative to enact desired physiological effects.

6. NO and its applications

NO has a very diverse chemical biology and function; for example, it has a role in vascular relaxation [90], has anti-thrombolytic and anti-inflammatory effects [91], is involved in neurotransmission, immune-response facilitation, has antipathogenic response [92–94], has a central role in angiogenesis and is a mediator of the vascular endothelial growth factor (VEGF) [95], it displays antiatherosclerotic properties [96], and has a dichotomous role cancer biology [97]. Many of its actions follow a biphasic dose-response, which ranges from physiological, cytoprotective effects at relatively low concentrations to cytotoxic effects at much higher concentrations, reviewed in [3, 98–101]. In the following sections, we have reviewed some of the means by which NO is utilized to meet a particular clinical need and means by which its delivery is manipulated.

6.1. Classical synthetic NO donors

Before discussing synthetic NO donors, it is worthwhile to note that the FDA approved NO inhalation for treatments of patients with acute respiratory distress syndrome (ARDS) and for newborns with pulmonary hypertension in 1999 [102]. NO inhalation was shown to be beneficial in patients with post-cardiac arrest, extracorporeal membrane oxygenation (ECMO), cardiopulmonary bypass (CPB), sickle cell disease (SCD), acute chest syndrome (ACS), and lung and heart transplants [102]. However, NO inhalation as a means of drug therapy has potentially serious side effects. These include formation of nitrite (NO_2^-), which can react with the alveolar lining fluid producing nitric acid; formation of peroxynitrite ($OONO^-$) if NO reacts with superoxide anions; reaction with oxyhemoglobin to yield methemoglobin leading to systemic hypoxemia, and others. These side effects limit the use

of NO inhalation as a viable therapeutic modality, and caution must be exercised when doing so.

Nitrovasodilators (Fig 8), which include nitroglycerine (glycerol trinitrate), amyl nitrite, isosorbide mono- and dinitrate, erythrityl tetranitrate, and sodium nitroprusside are medications that are taken sublingually, orally, or subcutaneously for the treatment of angina pectoris and other coronary artery diseases. Nitroglycerine has been used effectively for over 100 years, and the other organic medicinal nitrates have been available since the 1930s. Because NO has a dichotomous role in cancer biology, with some reports suggesting that NO possesses anti-tumor properties, while others implicate NO in tumor promotion, in theory, these medications can inhibit or promote the development of cancer. For example, isosorbide mononitrate and dinitrate were shown to inhibit angiogenesis, tumor growth, and metastasis in mice [103], while feeding glyceryl trinitrate to F344 rats induced hepatocellular carcinomas [104]. A novel NO donor, 3-morpholino-sydnonimine (SIN-1, Fig 3) and its analog, a dual-acting NO-releasing and reactive oxygen-scavenging hybrid compound SA-2, were shown to lower elevated intraocular pressure that is associated with degeneration of the optic nerve and loss of retinal ganglion cells [105] by increasing superoxide dismutase enzyme activity. A seminal review on synthetic NO donors is given by Wang et al [106].

6.2. Classical NO-NSAID conjugates

Considerable epidemiological, interventional, and animal studies have established nonsteroidal anti-inflammatory drugs (NSAIDs) as the prototypical chemopreventive agents against many forms of cancer [3, 25, 100, 101, 107–109]. Chronic NSAID use eventually causes some degree of gastrointestinal (GI) erosions, which eventually may lead to ulcers, with most having cardiovascular (CV) and renal side effects. In order to overcome these potential side effects, nitric oxide-releasing NSAIDs (NO-NSAIDs), also known as COX-inhibiting nitric oxide donors (CINODs) were developed [110, 111]. The rationale for their development was essentially based on the observations that within the GI system, NO can enhance the local mucosal defense mechanisms, offsetting the decreases in prostaglandins (PGs) that come about due to cyclooxygenase (COX) inhibition following chronic NSAID use [112]. NO-NSAIDs have safer GI profiles compared to their corresponding parent NSAID in animals [113–120] and humans [121, 122].

NO-NSAIDs are traditional NSAIDs linked to a NO-releasing group via a chemical spacer. The three key structural components of this class of NO-NSAID are: the traditional NSAID moiety; the spacer, which can be either aliphatic or aromatic; and the NO-releasing group, which initially was a nitrate ester as shown in Fig 9 A and B. In evaluating these NO-NSAIDs in cell culture against a variety cancer cell lines, it was shown that positional isomerism greatly influenced all cell kinetic parameters that influence cellular mass. For example, the *ortho* and *para* positional isomers of NO-aspirin were significantly more potent than the *meta* isomer, and when the spacer was aliphatic the activity was considerably lower [123–125].

A second generation of NO-releasing aspirins uses furoxan derivatives as NO donors [126], (Fig 9–C). Unlike the nitrate esters which required enzymatic metabolism for NO release

[127–129], the furoxan-based NSAID hybrids released NO in the presence of plasma, GSH, or albumin, that is through thiol-triggered mechanisms [130]. Another class of NO-releasing "aspirin-like" compounds have also been described where the acetyl group on the aspirin has been replaced by acyl groups containing nitroxy NO-releasing moieties (Fig 9 D and E). All these compounds have exhibited reduced GI toxicity compared to aspirin, and have strong anti-inflammatory properties [131].

6.3. NO-releasing coxibs

Selective cyclooxygenase-2 inhibitors (Coxibs) such as celecoxib, rofecoxib, and valdecoxib were developed to overcome the GI side effects of traditional nonselective NSAIDs [132], which are attributed to inhibition of COX-1. Overall, this class of compounds has a very good GI safety profile in the short term; however, this appears to be less robust with long-term use. However, total inhibition of COX-2 can lead to an eicosanoid imbalance by down-regulation of PGI_2 and unaffected levels of TXA_2 leading to increased chances of CV side effects events [133] as confirmed by several large-scale clinical trials, reviewed by [100, 134]. NO is cardioprotective in much the same way as PGI_2 and it also inhibits both platelet aggregation and adhesion. Coxibs that release NO do exhibit a safer CV profile as exemplified by VA 694 showing significant improvement of coronary flow and a reduction of endothelial dysfunction [135]. Some examples include NO-celecoxib [136], NO-rofecoxib [137], NO-valdecoxib [138], VA 694 [135] (Fig 10 A–D, respectively); some others such as (pyrazoyl)benzenesulfonamides are derivatives of celecoxib [139] (Fig 10E), and a diazen-1-ium-1,2-diolate [140] (Fig 10F, an example of a NONO-coxib). There are a number of newly described NO-coxibs that are at various stages of preclinical development [141–148]; these compounds have enhanced solubility and appear to be more potent. Therapeutic applications of these prodrugs are diverse.

6.4. Diazeniumdiolate-based NO-releasing compounds

Diazeniumdiolates (NONOates) are prodrugs that are revealed upon hydrolysis or metabolic activation form the parent NONOate anion, which further decomposes to release up to two moles of NO and the parent amine [149, 150] (Fig 11A). These prodrugs have an array of applications that are largely depend on the *O*-2 protecting group ('R', Fig 11A) and its mechanism of activation. Vinyl protected prodrug V-PYRRO/NO (Fig 11B) is activated by cytochrome P450 to release NO and shows hepatoprotective properties against a variety of toxins [151]. Glutathione (GSH)-activated arylated prodrug JS-K (Fig 11C) has anticancer activity [152–155]. Primary amine diazeniumdiolate prodrug AcOM-IPA/NO (Fig 11D) [156, 157] was reported to release nitroxyl (HNO) on protonation at N-2 (see Fig 11A for numbering); with possible applications in treating heart failure, alcohol abuse, and cancer [142, 158, 159]. Secondary amine diazeniumdiolate ions are protonated at N-3 to release NO [149].

6.5. NONO-NSAIDs

NONO-NSAIDs are based on linking a *N*-diazen-1-ium-1,2 diolate functional group to an NSAID and can potentially generate 2 equivalents of NO (Fig 11E). NONO-NSAIDs do not require redox activation before NO is released [160], whereas nitrate esters require a three-electron reduction [161]. The first agent reported in this class had a NONOate (O^2-

unsubstituted *N*-diazen-1-ium-1,2-diolate) attached via a one-carbon methylene spacer to the carboxylic acid group of a traditional NSAID (aspirin, ibuprofen, indomethacin) [162] (Fig 11A, R = (1) or (2)). The next series of NONO-NSAIDs (aspirin, ibuprofen, indomethacin) possessed an O^2-acetoxymethyl-1-[*N*-(2-hydroxyethyl)-*N*-methylamino]diazen-1-ium-1,2-diolate moiety as the NO donor (2-HEMA/NO) [160] (Fig 11A, R = 3). Because in their synthesis a secondary dialkyamine was used, this led to a number of possible new NONO-NSAIDs. Close inspection revealed that upon hydrolysis it was possible to release one equiv of the corresponding nitrosoamine, a biologically toxic compound. To overcome this concern, a second-generation of O^2-acetoxymethyl-protected (e.g., PROLI/NO) releasing NONO-NSAIDs was developed where a diazeniumdiolate ion obtained from an amine such L-proline, was used, the *N*-nitroso derivative of which is nontoxic [163] (Fig 11A, R = 4). As a class, all NONO-NSAIDs are reported to be devoid of GI toxicity, with no inhibitory effects on either COX-1 or COX-2, but have potent anti-inflammatory properties, consistent with acting as prodrugs requiring metabolic activation to release the parent NSAID.

6.6. HNO-NSAIDs

Decomposition of diazeniumdiolates can lead to formation of nitroxyl (HNO) and/or NO [149]. Potential actions of HNO are in overcoming heart failure [159], preconditioning against myocardial infarction [164], and treating alcohol abuse [158]. Using Angeli's salt ($Na_2N_2O_3$) to generate HNO, the first anticancer activity of HNO was reported in 2008 [165]. Recently two new NONO-NSAIDs were prepared by derivatizing both a primary and secondary amine diazeniumdiolate with aspirin to produce O^2-(acetylsalicyloyloxymethyl)-1-(*N*-isopropylamino)-diazen-1-ium-1,2-diolate (IPA/NO-aspirin) and O^2-(acetylsalicyloyloxymethyl)-1-(*N*,*N*-diethylamino)-diazen-1-ium-1,2-diolate (DEA/NO-aspirin) [166] (Fig 11A). Both have shown enhanced GI safety profiles, strong anti-inflammatory properties, and significantly enhanced cytotoxcity compared to either aspirin or the parent diazeniumdiolate toward nonsmall cell lung carcinoma cells (A549), but were not toxic toward endothelial cells (HUVECs) suggesting cancer-specific sensitivity.

6.7. JS-K and PABA/NO

The diazeniumdiolate prodrugs, JSK [O^2-(2,4-dinitrophenyl)1-[(4-thoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate] (Fig 11C) and PABA/NO [O^2-[2,4-dinitro-5-(*N*-methyl-*N*-4-carboxyphenylamino) phenyl] 1-*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate] (Fig 11F) were designed to be activated as anticancer agents by glutathione-*S*-transferase (GST)-induced release of NO [167]. The rationale for this was based on the observation that GST (specifically GST-π, a key phase II detoxification enzyme, is frequently over-expressed in cancer tissue [168, 169]. JS-K [155, 168, 170–173], and PABA/NO [174–176] have shown promise as anti-cancer agents. In order to improve the selectivity for cancer cells, hybrids of O^2-(2,4-dinitrophenyl)diazeniumdiolates and oleanolic acid (OA) have been prepared [177], (Fig 11G). The rationale for these hybrids was based in part on the observation that OA imparts additional hepatic selectivity and a synergetic biological profile to the GSTπ-activated moiety [178], reviewed in [101].

6.8. RRx-001: an NO modulatory anticancer agent

RRx-001 (Fig 12B) also known as ABDNAZ (1-bromoacetyl-3,3-dinitroazetidine) is a novel aerospace-derived compound under active investigation as a chemo-, immuno-, and radiosensitizer. RRx-001 demonstrated antitumor activity and minimal toxicity in phase II clinical trials and has received clearance from the FDA and the EMA for phase III, multicenter studies in subjects with relapsed/refractory solid tumors (Clinical Trial registration: NCT03699956) [179–181]. This compound contains a unique, highly energetic organic nitro functional group called a gem dinitroazetidine that has not been used to date for medical and pharmaceutical applications. In an aerospace setting, compounds containing this energetic functionality, such as 1,3,3 trinitroazetidine, are designed to fragment explosively to propel rockets [182]. Modification of this structure by removing one of the nitro groups and substituting it with a bromoacetate group resulted in RRx-001, a nonexplosive that may be used to treat cancer [183], reviewed in [101]. RRx-001differs from other NO-donating compounds in that the molecule induces local, endogenous, and biphasic production or release of NO, rather than fragmenting to release NO systemically. This activity is closely linked to the metabolism of RRx-001; on infusion, the compound rapidly, irreversibly, and selectively binds to hemoglobin at a key NO binding site [184], and with glutathione [185, 186] in directly increasing oxidative stress [187]. While the RRx-001 glutathione adduct is rapidly excreted, RRx-001-bound hemoglobin remains in circulation for the duration of the lifetime of the red blood cell [188].

6.9. Light triggered NO donors

Photodynamic therapy (PDT) is a novel approach for treatment of various pathologies including cancer and infectious diseases. The therapy is based on the interaction of a photosensitizer (PS), light and oxygen. None of these is individually toxic, but the combination produces a photochemical reaction that leads to the production of ROS and/or singlet oxygen ($^1O_2$) [189]. Cell death then occurs by apoptosis, autophagy or necrosis and the outcome depends on the PDT dose and localization of the PS [190, 191]. PDT is a 2-stage procedure. After the administration of a light-sensitive PS, tumor loci are irradiated with a light of appropriate wavelength that can be delivered to virtually any organ in the body by means of flexible fiber-optic devices [191]. Most PSs used in cancer therapy belong to the protoporphyrin family and are based on a tetrapyrrole structure [192]. An ideal sensitizer must have an absorption peak between 600 and 800 nm (red to deep red), higher wavelengths do not have enough penetration and do not excite oxygen to its singlet state, thus reducing generation of ROS that are required for cytotoxic effects [193].

The NO influence the response of the tumor cells to PDT [194, 195]. Activated PS can induce the production of NO by increasing the expression of constitutive NOS [196] or that of iNOS [197], and as discussed in this review and elsewhere [3, 100] NO has a dual role in cancer biology.

6.9.1. NO donors and PDT—This is an area of intense interest for medicinal chemists and its potential applications for cancer therapeutics. Many groups have synthesized NO donors to promote PDT-mediated anti-tumor cytotoxicity. Examples of photochemical NO releasing compounds incorporating various transition metals are, Roussin’s red salt anion

$[Fe_2S_2(NO)_4^{2-}]$, and a ruthenium-nitrosyl complex where the NO is caged by coordination to the transition metal center; and a Cr(III) nitrito complex where the NO is caged by bonding to another oxygen, releasing NO by homolytic cleavage of the MO–NO bond, the chemistry of these compounds has been reviewed by Ford [198]. Another PS of interest is a silicon-phthalocyanine compound (Pc4) [196]. Recently, a series of photo responsive *N*-nitrosoaniline based NO donor polymers [amphiphilic diblock copolymers, PEO_{45}-*b*-P*o*NBN_{25} (BP1), PEO_{45}-*b*-P*p*NBN_{30} (BP2), and PEO_{45}-*b*-PBN_{46} (BP3)] were synthesized by nitrosation of 4-aminobenzyl alcohol-based precursors [199]. Using appropriate probes, photo-mediated NO release from BP1 vesicles was confirmed in HeLa cells. Furthermore, BP1 was shown to be effective in a corneal wound-healing model.

6.9.2. Clinical applications of PDT—Ocular infection due to microbial contamination is one of the main risks associated with the wearing of contact lenses. Recently an NO-releasing soft contact lens that releases NO under daylight exposure was reported to be safe and have good activity against of *Staphylococcus aureus* [200]. PDT has been effectively used to treat Bowen's Disease (BD), also known as squamous cell carcinoma in situ (SCCis), which most often is caused by exposure to ultraviolet light but may also occur as a result of Human Papillomavirus, arsenic exposure, or chronic radiation dermatitis [201]. Studies have shown that PDT is equally or more effective than conventional therapies such as 5-fluorouracil and cryotherapy in treating BD [202]. PDT has been used to treat cancers of the head and neck, prostate, bladder, lung, skin, gastrointestinal tumors, intraperitoneal malignancies, and others have, reviewed in [191].

6.10. Dual NO-H_2S donors

Recently, a new class of anti-inflammatory pharmaceuticals were described in which an NO-releasing and an H_2S-donating moiety were covalently attached to an NSAID backbone, thus releasing both NO and H_2S; these chimeras have been termed NOSH-NSAIDs [203, 204] (Fig 13). The rationale for their development was based on the chemistry of NO and H_2S, and the structural components of NO-NSAIDs and H_2S-NSAIDs thus postulating that a hybrid capable of releasing both of these gasotransmeters might be more potent and effective than either one alone [203]. A number of these compounds have been reported; nitrate was used for NO release and this was attached to the parent NSAID through an aliphatic spacer, while one of the following moieties, 5-(4-hydroxyphenyl)-3H-1, 2-dithiole-3-thione (ADT-OH), or 4-hydroxy benzothiazamide (TBZ) or lipoic acid were used for H_2S release. NOSH-NSAIDs displayed greater GI safety profiles compared to their parent counterparts and displayed strong antioxidant properties [205–207]. NOSH-aspirin, (NBS-1120) exhibited strong anti-inflammatory [203, 206], anti-pyretic, analgesic, and antiplatelet properties similar to its parent compound, aspirin [206]. NOSH-aspirin inhibited the growth of eleven different human cancer cell lines of six different histological subtypes with IC_{50}s that were in the low to mid nanomolar ranges [203]. Using HT-29 colon cancer cells as a model, this growth inhibition was as a result of reductions in cell proliferation, G_0/G_1 cell cycle arrest, leading to increased apoptosis [208]. The efficacy of NOSH-aspirin at different concentrations was also compared to that of aspirin using an *in vivo* xenograft mouse model of colon cancer chemoprevention [206]. NOSH-aspirin dose-dependently inhibited tumor growth, and tumor mass and was at least 5-fold more potent than aspirin. Of

note, NOSH-aspirin was also efficacious against established tumors in a xenograft model of colon cancer [208]. Clearly, as a chemo-preventive and chemotherapeutic agent, NOSH-aspirin is superior to aspirin both in terms of efficacy and safety. Qualitatively similar results have been reported for both NOSH-naproxen [207] and NOSH-sulindac [205]. With regards to efficacy of NOSH-naproxen in a xenograft model of colon cancer, it is important to note that while treatment of animals with NOSH-naproxen significantly reduced tumor growth and tumor mass with no overt sign of GI toxicity, naproxen-treated mice died due to GI bleeding [207]. Interestingly, when examining cell growth inhibition in the presence of the three individual components of NOSH aspirin (ADT-OH, a small molecule NO donor, and aspirin), the cocktail had an IC_{50} of 450 μM, a 9,000-fold difference compared with that of the intact NOSH-aspirin. These results indicate that cancer cell growth inhibition is influenced by more than simply delivering DTT and NO concurrently with aspirin, but the reasons for this synergy, although largely unknown, may have to do with generation of more potent entities such as persulfides.

Apart from being active against cancer and having potent anti-inflammatory properties, NOSH-aspirin (NBS-1120) and NOSH (NBS-1100, a molecule where butyl nitrate and ADT-OH are directly linked together, Fig 13) have protective effects in drought-stressed *Medicago sativa* L. Plants [209]. Plants were pre-treated with NOSH or NOSH-aspirin by foliar spraying and then exposed to moderate water deficit, while NO and H_2S inhibitors (cPTIO and HT, respectively) were also employed. Phenotypic and physiological data showed that pre-treatment with the NOSH chimeras induced acclimation to subsequent drought stress and improved recovery following rewatering. This was accompanied by modified reactive oxygen and nitrogen species signaling and metabolism, as well as attenuation of cellular damage as evidenced by altered lipid peroxidation and proline accumulation levels. Furthermore, real-time RT-qPCR analysis revealed the differential regulation of multiple defense-related transcripts including enzymatic antioxidants [209].

Another H_2S-NO hybrid molecule (ZYZ-803, Fig 13) has been shown to have efficacy in isoprenaline-induced heart failure [210]. The cardioprotective effect of ZYZ-803 was more potent than that of the H_2S and/or NO donor alone. ZYZ-803 increased expression of CSE and eNOS activity. Blocking CSE and/or eNOS suppressed ZYZ-803-induced H_2S and NO production and cardioprotection. ZYZ-803 increased VEGF and cGMP levels and also upregulated the endogenous antioxidants glutathione peroxidase (GPx) and heme oxygenase1 (HO-1). ZYZ-803 also induced angiogenesis in human umbilical vein endothelial cells (HUVECs) with STAT3 as well as CAMKII in mediating this effect [211].

7. Engineered NO delivery platforms

Delivering NO in a temporally and spatially controlled fashion can be challenging because of its potential release from the conjugated system in the first few minutes after administration. Of concern is also that some of the metabolic decomposition products can potentially be toxic [212]. To overcome these limitations and others, many different delivery platforms have been developed; some include polymers, nanoparticles, liposomes, dendrimers, and porous materials [213–219] (Figure 7).

7.1. Polymers

Bio-polymeric scaffolds should be non-toxic, biodegradable, and biocompatible. They can be either naturally occurring, such as sugar-based materials including chitosan, dextran, and hyaluronic acid [220–224], or synthetic polymers such as dendrimers. Polymer-based materials that are used for NO delivery could be used as coatings, films, or ointments. These polymers may contain RSNOs, nitrosamines, NONOates, or other NO-releasing entities, which overcome the limitations of their therapeutic use. This mode of NO delivery allows for controlled kinetic release, which may last for days [225] or even weeks [226]. The most common preparation strategy is based on dispersing thiol- or amine containing compounds in polymers, followed by exposure to NO gas to convert the parent polymer materials into NO donors [227]. Matrix structures have been used for functionalization with NO-releasing molecular systems because of their great biocompatibility with living tissues [228, 229].

Dendrimers are globular structures that consist of a central core surrounded by a highly branched corona with reactive surface groups; they are a particularly attractive class of synthetic polymers because of their multivalent surface and well-defined polymeric structure [230]. Because of their exterior functional groups encompassing a steric environment and hydrophobicity, multiple derivative structures are possible with varying NO payloads [231].

7.2. Nanomaterials

Due to their small size, nanomaterials have enhanced interaction and tissue penetration. Mesoporous silica nanoparticles (MSN) are biocompatible systems that have been used as NO carriers with a number of NO-releasing compounds including diazeniumdiolates [232]. Gold [233–235], silver [236, 237], and iron oxide nanoparticles [238–240], as well as quantum dots [241, 242], are other NO nanotechnology platforms that may be used for both diagnostic and therapeutic applications [227]. The preparation of these complexes is based on their surface functionalization with diazeniumdiolate or *S*-nitrosothiol groups [235, 238, 243]. Hollow polymeric nanoparticles made of synthetic polymers (e.g., polymethacrylate and polydopamine) have unique properties such as low density, optical scattering, and good flow capacity [244]. The large surface area of hollow polymeric nanoparticles facilitates NO donor functionalization on both the inner and outer surfaces, leading to larger NO payloads [245].

7.3. Liposomes

Liposomes are spherical vesicles composed of an inner aqueous core and a phospholipid bilayer outer shell, which can be either synthetic or natural, with an overall structure that mimics natural cell membranes. Both NO gas and NO donors (e.g., *N*-diazeniumdiolates, metal nitrosyls, and organic nitrites) have been encapsulated into liposomes to achieve controlled NO release [245–247]. The main techniques employed in forming liposomes are thin-film hydration, solvent injection, reverse-phase evaporation, membrane extrusion, and microfluidic technology [248, 249]. NO-releasing liposomes have great potential to be used for anticancer therapy [250, 251].

7.4. Porous materials

Porous materials for NO storage/release cover a large variety of structures that can be organic, inorganic, and inorganic-organic hybrids with varying pore sizes, percentage of porosity, and the presence (or absence) of interconnectivity between them [227, 252]. These include zeolites, titanosilicates, clays, and MOFs (metal organic frameworks). Chemisorption or physisorption is used to store NO with a wide range of metal ions within the pores, and NO is then released when water replaces NO on the metal centers and diffuses out of the porous structure. This methodology provides a highly efficient packing of NO within the solid and provides for controlled delivery to target tissues [227, 253, 254].

Zeolites are highly crystalline aluminosilicate microporous insoluble materials with a rigid three-dimensional open framework that may be of natural or synthetic origin [227]. Exposure to NO gas can result in both reversible and irreversible NO adsorptions, giving rise to different release kinetics. Also, the affinity for NO of the transition metals used in the various zeolites greatly affects the extent to which NO is adsorbed and released. Studies with zeolites Linde type A and Faujasite with Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+} showed that Co had the highest storing capacity [255]. Topical application of NO-releasing zeolites (0.02 mL 33%, wt/wt) induced local vasodilatation and no significant inflammatory response [256]. Application of a topical ointment containing NO-loaded zinc-exchanged zeolites to wounds three times per week for 20 days in Zucker obese rats resulted in enhanced wound healing [257]. Furthermore, in vitro microbial studies showed activity against *Escherichia coli, Acinetobacter baumannii, Staphylococcus epidermidis*, methicillin-resistant *S. aureus* (MRSA), and *Candida albicans* fungus. An NO-releasing Zn^{2+}-exchanged zeolite at a 50 wt.% composition in a polytetrafluoroethylene polymer showed activity against both Gram-negative *Pseudomonas aeruginosa* and Gram-positive methicillin-sensitive and methicillin-resistant *S. aureus* and *Clostridium difficile* [258].

Titanosilicates are microporous zeolite-type silicates possessing framework of unsaturated transition-metal centers; examples include ETS-4 [$Na_9Si_{12}Ti_5O_{38}(OH)\cdot xH_2O$], a titanosilicate that displays excellent NO adsorption capacity and slow releasing kinetics [254]. The use of this mode of NO delivery as applicable to biological systems needs to be investigated further.

Clays are already being used in the therapeutic field either as active substances or as excipients to other drugs [259]. They are very amenable due to their high mechanical, thermal, and chemical stability; they have regular structures and appreciable surface areas and thus have good adsorption and penetrability properties. NO-releasing mineral clays such as sepiolite and montmorillonite (MMT), synthetic clays (smectite clays with cobalt ions), and organo-clays (natural clays modified with L-histidine) have been developed and their activity evaluated in HeLa cells [260–262].

MOFs are porous hybrid inorganic-organic crystalline materials, built on metal ion oxoclusters connected by organic ligands, in a quasi-infinite array [263]. These are relatively new materials with varying toxicities due to the metals used and the amount of NO released. However, as a class, these are interesting and much more work is needed in order to make

them viable/suitable for medical applications. A detailed review of these compounds is covered elsewhere [227, 263].

7.5. Hydrogels

As noted in section 5.3, gels are non-fluid polymer or colloidal networks that are expanded by a fluid. If the expanding fluid is water, the gel is then called a hydrogel. Hydrogels can absorb more than 90% of their dry weight in water, while chemical and physical crosslinkingof the polymeric chains make them insoluble in water [229]. NO-releasing hydrogels have been prepared that incorporate different NO-releasing moieties, such as *S*-nitrosothiols, *S*-nitrosoglutathione, diazeniumdiolates, sodium nitrite, and others; and varying polymeric matrices, for example pHEMA coated with polyurethane, pluronic F127, PVA functionalized with –SNO groups, hydroxyethyl cellulose etc., have many therapeutic applications ranging from bactericidal, topical vasodilation, and wound healing [264–267] (for detailed review see [229]). There are also NO donors that release NO exclusively under irradiation, and several photoactive metal–nitrosyl complexes using a polymerized matrix of poly(2-hydroxyethyl methacrylate) (pHEMA) have been developed [266].

8. Conclusions

Significant progress has been made in the fields of NO and H_2S donor chemistry. Continued innovation from synthetic chemists will be a major factor in driving the NO~H_2S research forward in the coming years, with an eye toward building NO and H_2S donors that are clinically relevant therapeutics. Continued development of various delivery platforms for targeted therapy is of significant importance, with an additional focus towards further improving and developing platforms that are biocompatible in many contexts and degrade to form non-toxic metabolites. In this regard, the use of polymeric platforms is attractive due to low productions costs and ease by which different synthetic moieties can be incorporated. For example, polymeric particles can be functionalized with a poly(ethylene glycol) corona, giving rise to “stealth” properties which significantly increase circulation time and improved accumulation in tumors by the enhanced permeability and retention (EPR) effect [219]. While there are a considerable number of engineered platforms for the delivery of exogenous NO and H_2S, not much has been done on materials that may respond to these endogenous gasotransmitters. Thus NO and H_2S capturing materials may provide an unexplored area for clinical investigation.

PDT was the first drug-device combination approved by the FDA almost 3 decades ago, but it is underutilized clinically. The highly localized nature of PDT is one of its current limitations, because the treatment is ineffective against metastatic lesions, which are the most frequent cause of death in cancer patients. Ongoing research is focused on finding optimal PDT conditions to induce systemic immunity that may address this significant shortcoming [191]. For all of these efforts to flourish, it is important for chemists, pharmacologists, biologists, and physicians to work together. An era of precision medicine to improve individual outcomes is not too far in the future.

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Abbreviations:

3-MST	3-Mercaptopyruvate sulfurtransferase
ADT-OH	5-(4-Hydroxyphenyl)-3*H*-1,2-dithiole-3-thione
ARDS	Acute respiratory distress syndrome
BODIPY	Boron-dipyrromethene
CaMKII	Ca^{2+}/CaM-dependent protein kinase II
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
COX	Cyclooxygenase
cGMP	Cyclic guanosine monophosphate
CSE	Cystathionine γ-lyase
DTT	1,2-Dithiole-3-thiones
eNOS	Endothelial nitric oxide synthase
GI	Gastrointestinal
H_2S	Hydrogen sulfide
LPS	Lipopolysaccharides
LR	Lawesson's Reagent
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	Neuronal nitric oxide synthase
NA	Noradrenaline
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitroreductase
Nrf2	Nuclear factor-like 2
NSAIDs	Nonsteroidal anti-inflammatory drugs

PDE5A	Phosphodiesterase 5A
PGI_2	Prostacyclin
pHEMA	Poly(2-hydroxyethyl methacrylate)
Pluronic F127	Poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) triblock copolymer
ROS	Reactive oxygen species
RSNO	S-nitrosothiols
RSSH	Persulfides
SATO	S-aroylthiooxime
sGC	Soluble guanylate cyclase
SNAP	*S*-Nitroso-*N*-acetyl-penicillamine
SNP	Sodium nitroprusside
STAT3	Signal transducer and activator of transcription 3
TBZ	4-Hydroxy benzothiazamide
TML	Trimethyl lock
TXA_2	Thromboxane A_2
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

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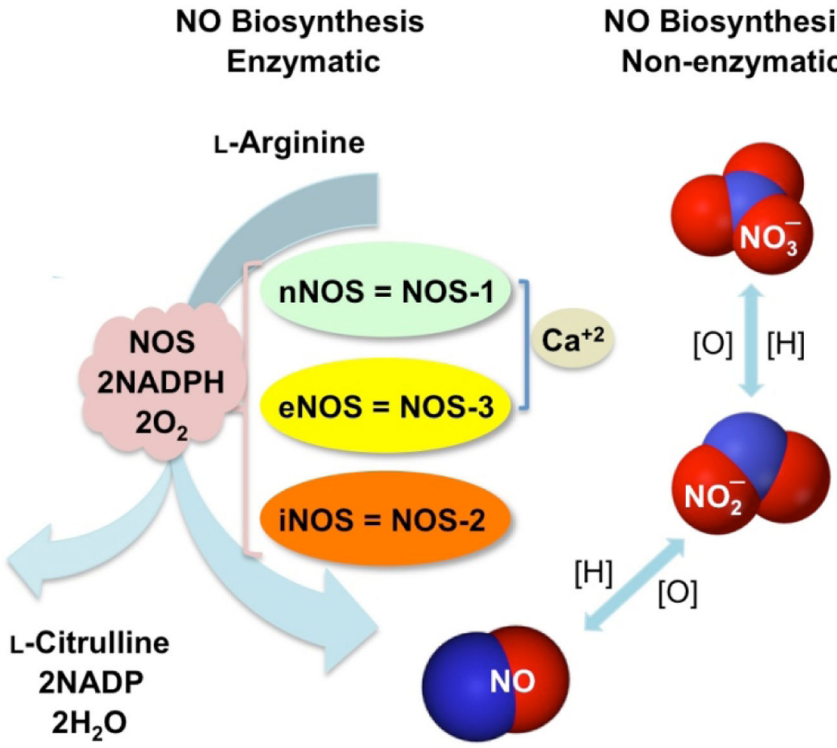

Figure 1.

Biosynthesis of nitric oxide. NO is produced by three nitric oxide synthase (NOS) isoforms: neuronal, endothelial, and inducible (nNOS, eNOS, and iNOS) that catalyze the oxidation of L-arginine to L-citrulline, the enzymatic pathway. NO is also produced through reduction of nitrite/nitrate under low oxygen conditions, the non-enzymatic pathway. [O] = oxidation, and [H] = reduction.

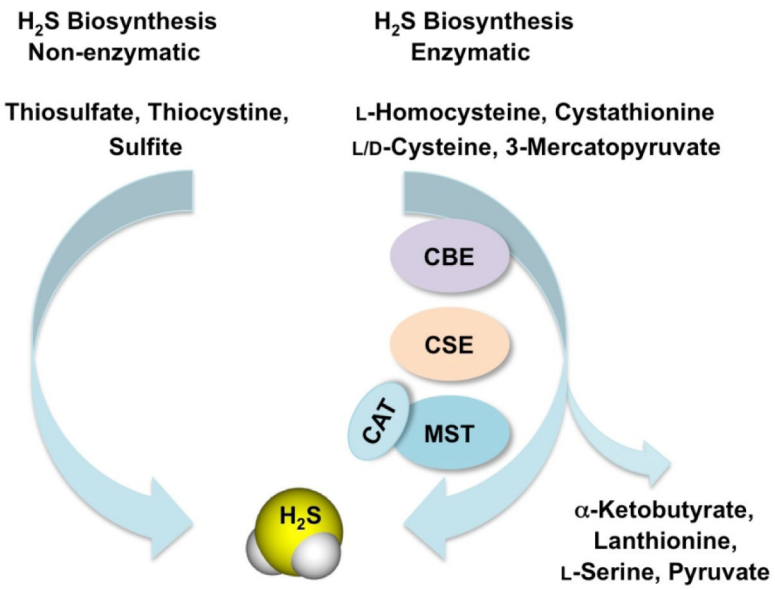

Figure 2.

Biosynthesis of hydrogen sulfide. H_2S is generated from oxidation of the substrates L-homocysteine, cystathionine, L-cysteine and 3-mercaptopyruvate through the enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) and the tandem enzymes cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST). α-Ketobutyrate, lanthionine, L-serine and pyruvate are the secondary products formed. Mammalian enzymes generally metabolize L-amino acids, however, H_2S can also be synthesized from D-cysteine by the peroxisomal enzyme D-amino acid oxidase (DAO) to 3-MP, which is a substrate for 3-MST. Alternatively, production of H_2S occurs non-enzymatically from various storage forms of sulfur such as thiosulfate, thiocysteine and sulfite.

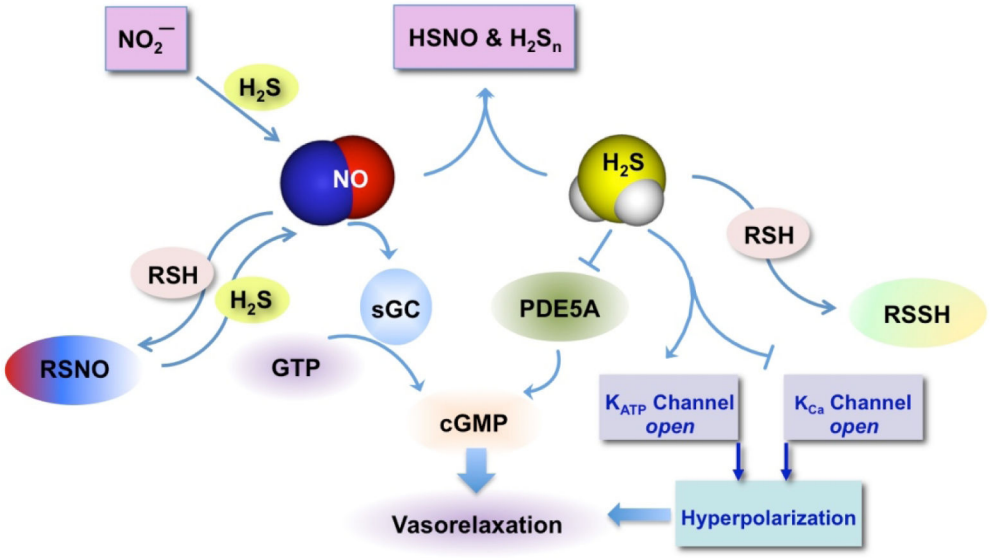

Figure 3.

Cellular effects of NO and H_2S and their interactions. NO reacts with the active site of soluble guanylate cyclase (sGC) and produces cyclic GMP leading to vasorelaxation. NO can affect cellular proteins by producing peroxynitrite, which in turn can interact with cysteine residues to form *S*-nitrosothiols (RSNO). The oxidative pathway leads to modification of proteins by *S*-nitrosylation of cysteine residues. H_2S raises cGMP levels through inhibition of phosphodiesterase 5A (PDE5A) an enzyme that catabolizes it. H_2S can also interact with the sulfhydryl group of cysteines and proteins to form persulfides (R-SSH). NO can interact with H_2S to form HSNO [32] and H_2S_n [45]; H_2S can interact with NO_2^- [34] or with RSNO [268–270] to produce NO. H_2S can interact with membrane ion channels and/or voltage-dependent calcium channels leading to vasorelaxation in vascular smooth muscle [26].

K_{ATP} = ATP-sensitive potassium, K_{Ca} = voltage-dependent calcium channels

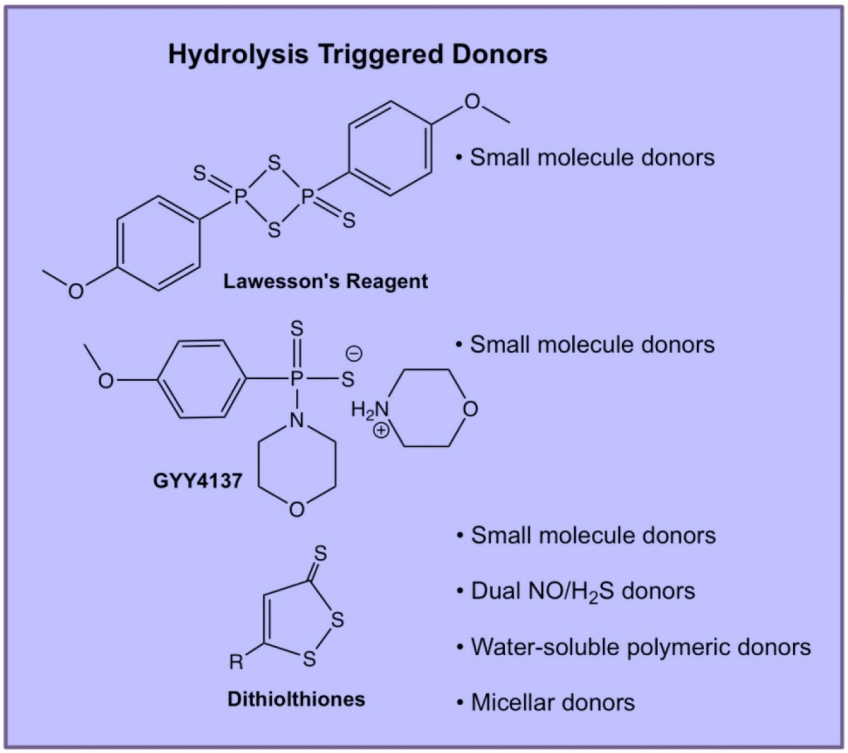

Figure 4.

Chemical structures of selected hydrolysis-triggered H_2S donors and descriptions of their use in various engineered delivery systems.

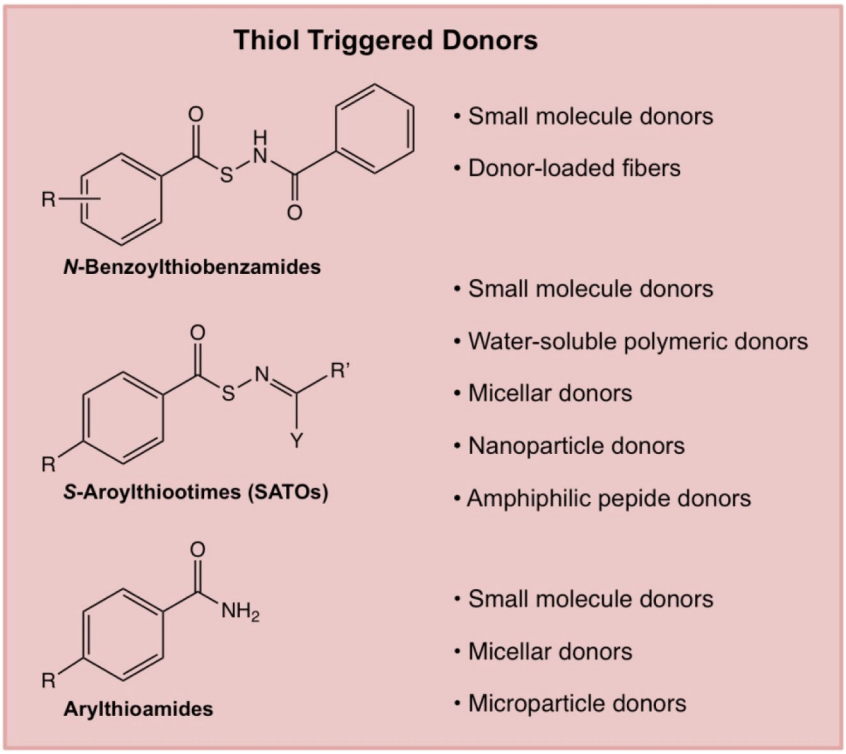

Figure 5.

Chemical structures of selected thiol-triggered H_2S donors and descriptions of their use in various engineered delivery systems.

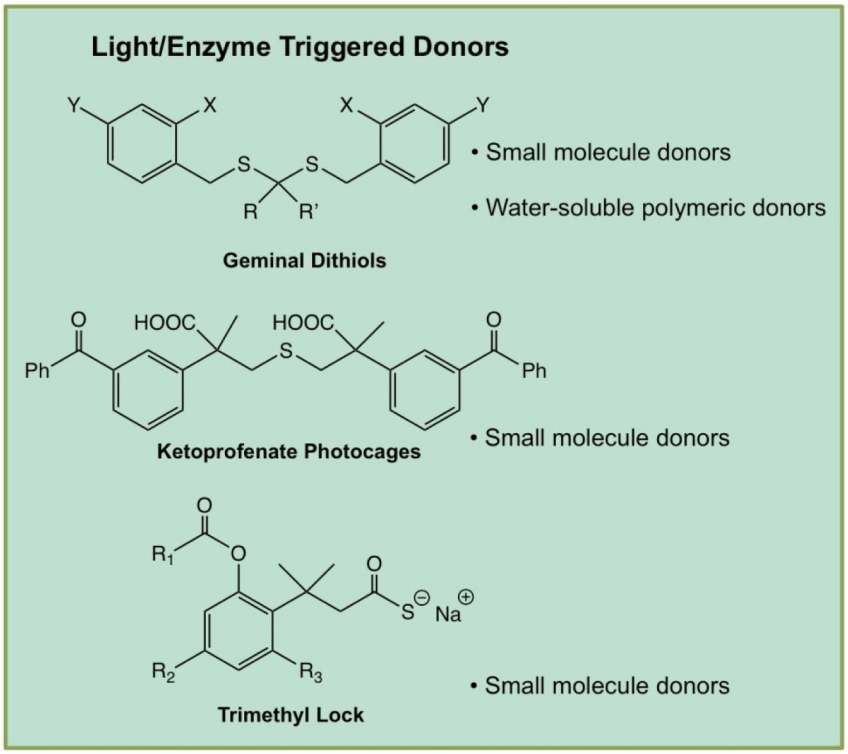

Figure 6.

Chemical structures of selected light- and enzyme-triggered H_2S donors and descriptions of their use in various engineered delivery systems.

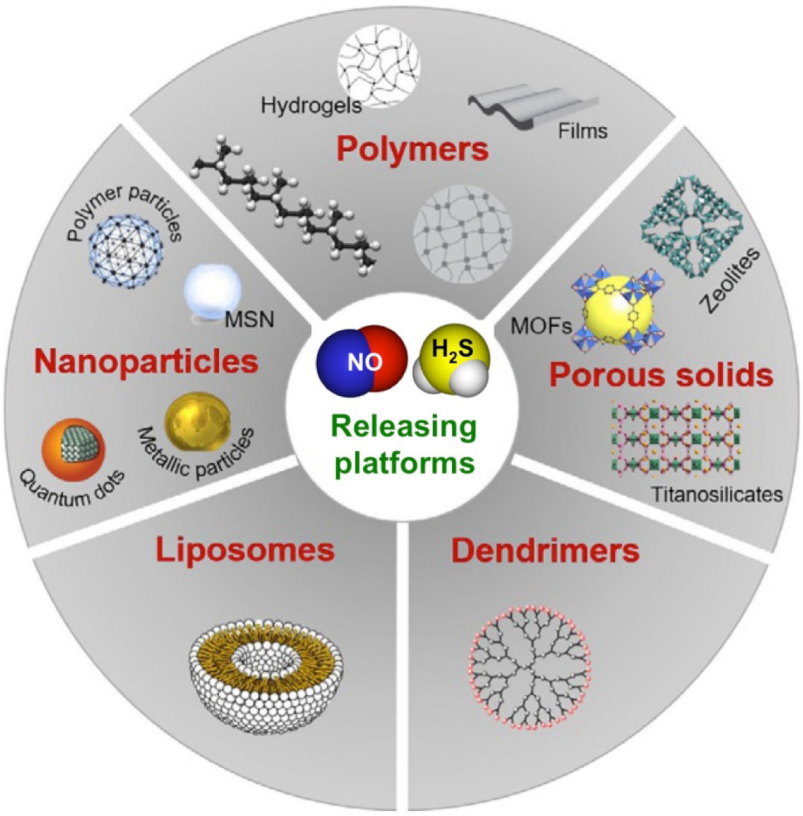

Figure 7.

A schematic summary of the different platforms that have been engineered to store and release NO and H_2S. *Adapted from Therapeutic Application of Nitric Oxide in Cancer and Inflammatory Disorders, by Rosana Vieira Pinto and Moises Luzia Pinto, in Nanoporous Materials: New Generation of Nitric Oxide Donors, Pages 277–304. Copyright (2019), with permission from Elsevier".*

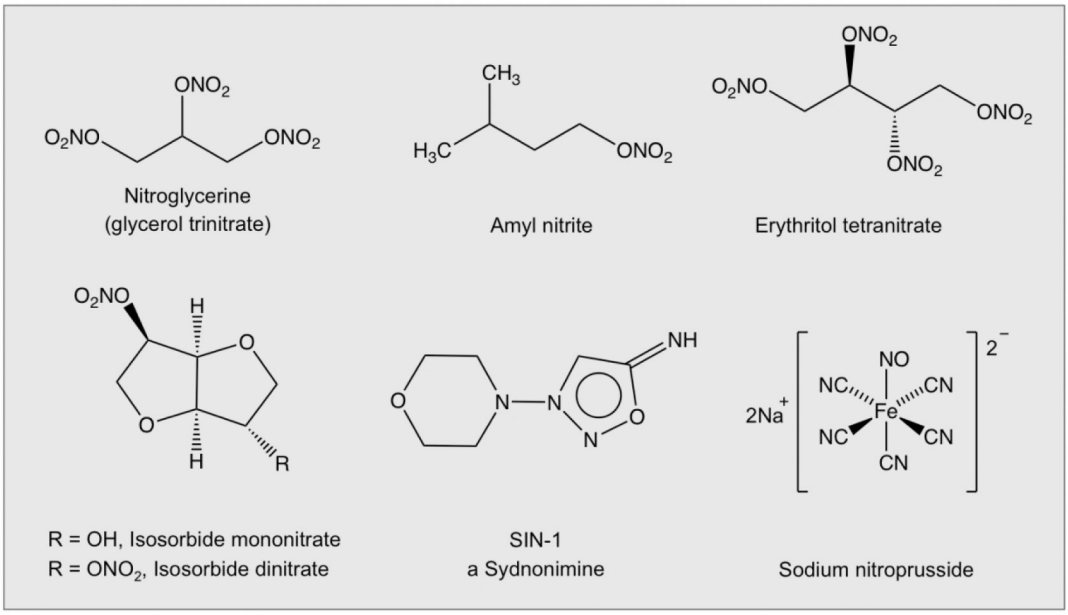

Figure 8.
The chemical structures of some nitrovasodilators.

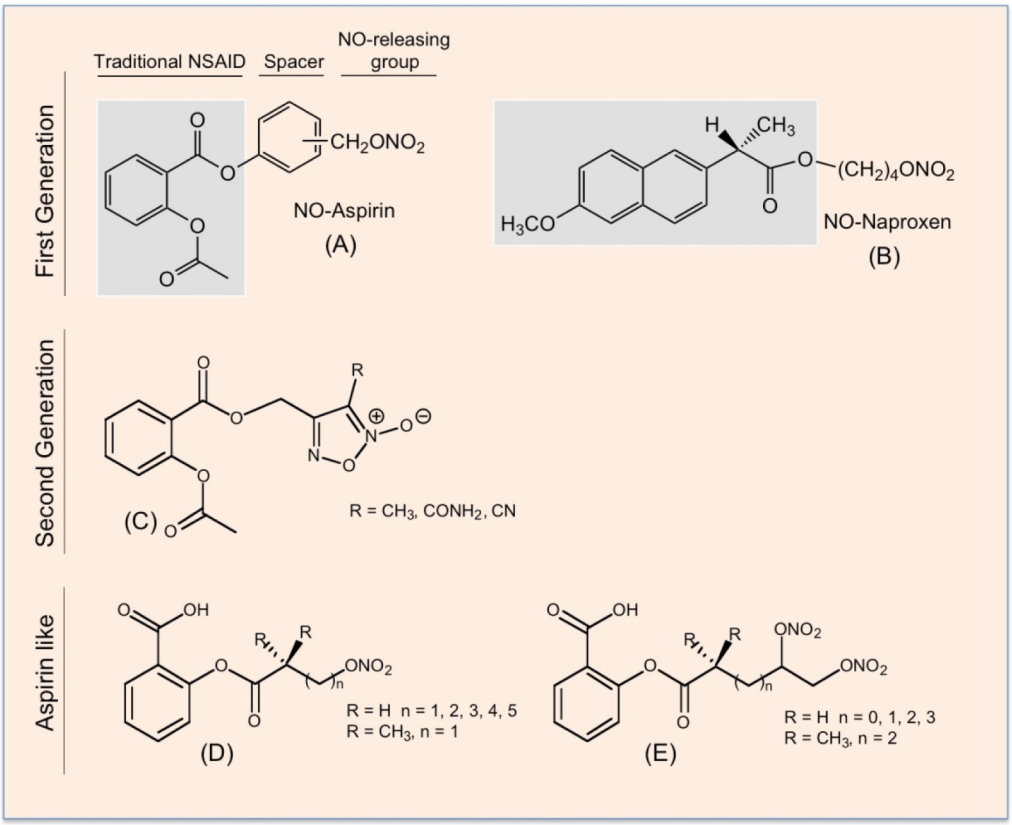

Figure 9.

The chemical structures of first- and second-generation NO-NSAIDs and NO-donor "aspirin-like" compounds. The traditional NSAIDs, aspirin (A) and naproxen (B), are shown in the shaded boxes; the spacer molecule links the traditional NSAID to $–ONO_2$, which can release NO. A second generation of NO-releasing aspirin in which a furoxan derivative is the NO donor (C). In the "aspirin-like" compounds, the acetyl group on the aspirin has been replaced by acyl groups containing nitroxy NO-releasing moieties, (D) and (E).

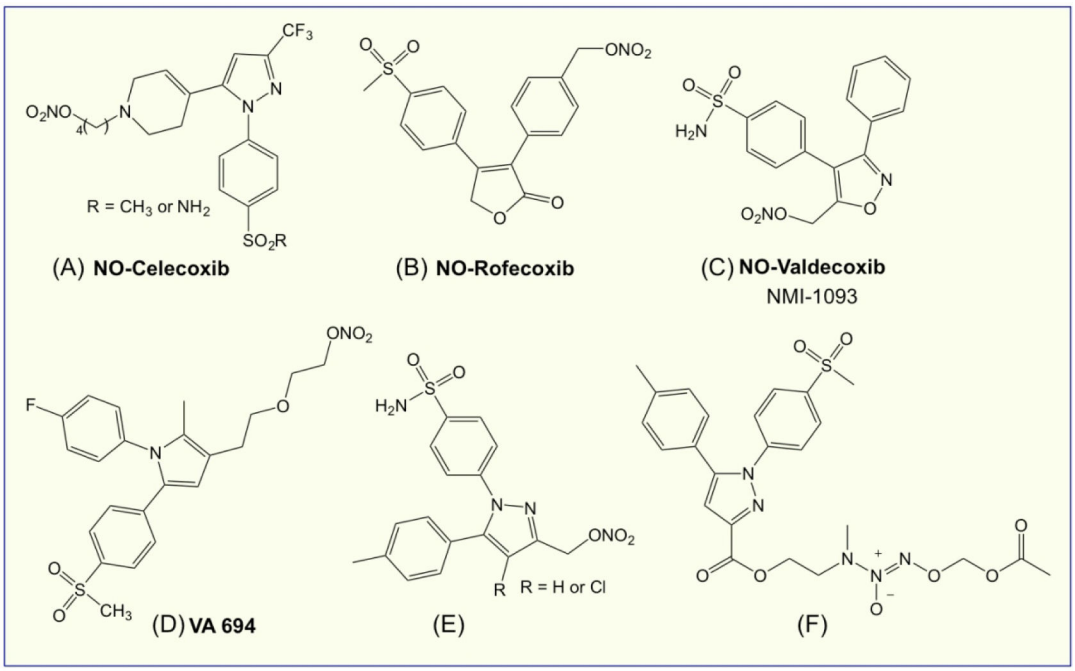

Figure 10.
The chemical structures of some NO-coxibs.

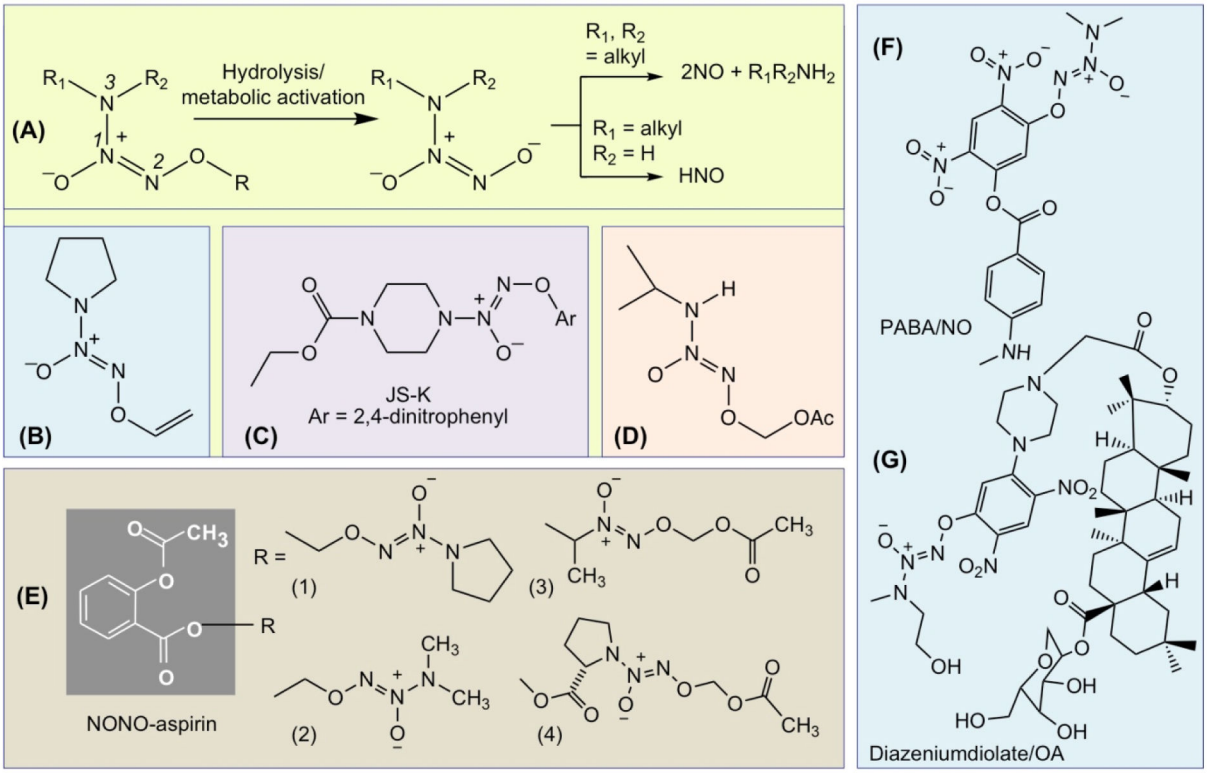

Figure 11.

Activation of diazeniumdiolate prodrugs to release NO or HNO (A). Structures of V-PYRRO/NO (B), JS-K (C), and AcOM-IPA/NO (D). The chemical structures of NONO-aspirin (E); PABA/NO (F), and diazeniundiolate/OA hybrid (G).

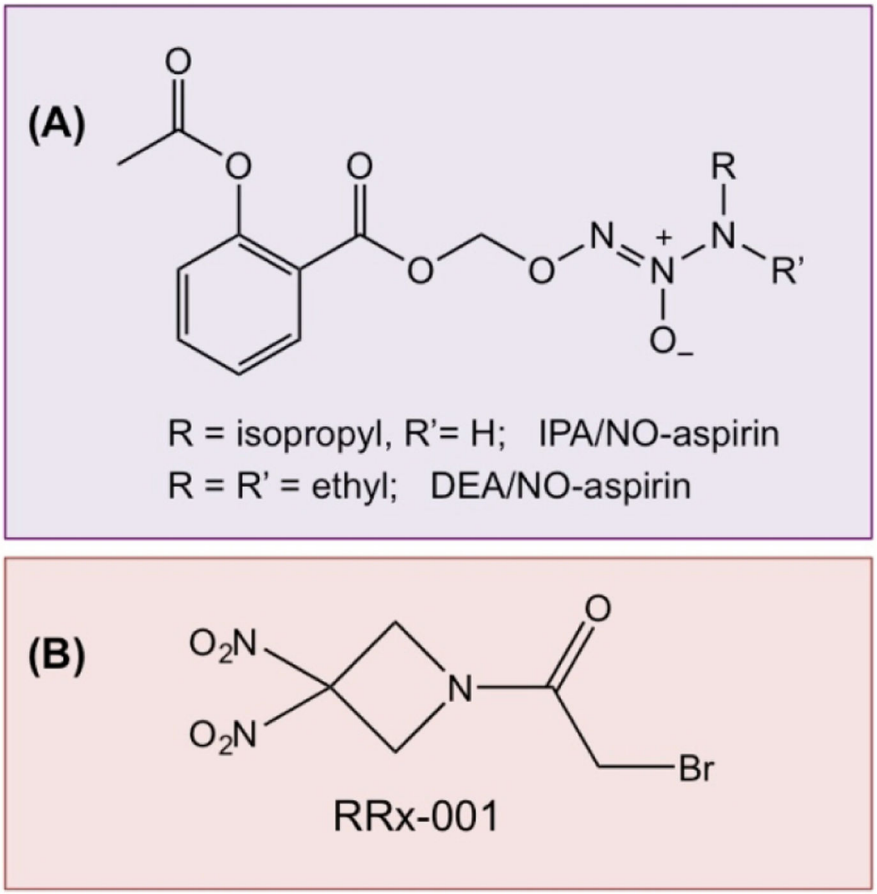

Figure 12.
The chemical structures of IPA/NO-aspirin and DEA/NO-aspirin (A); RRx-001 (B).

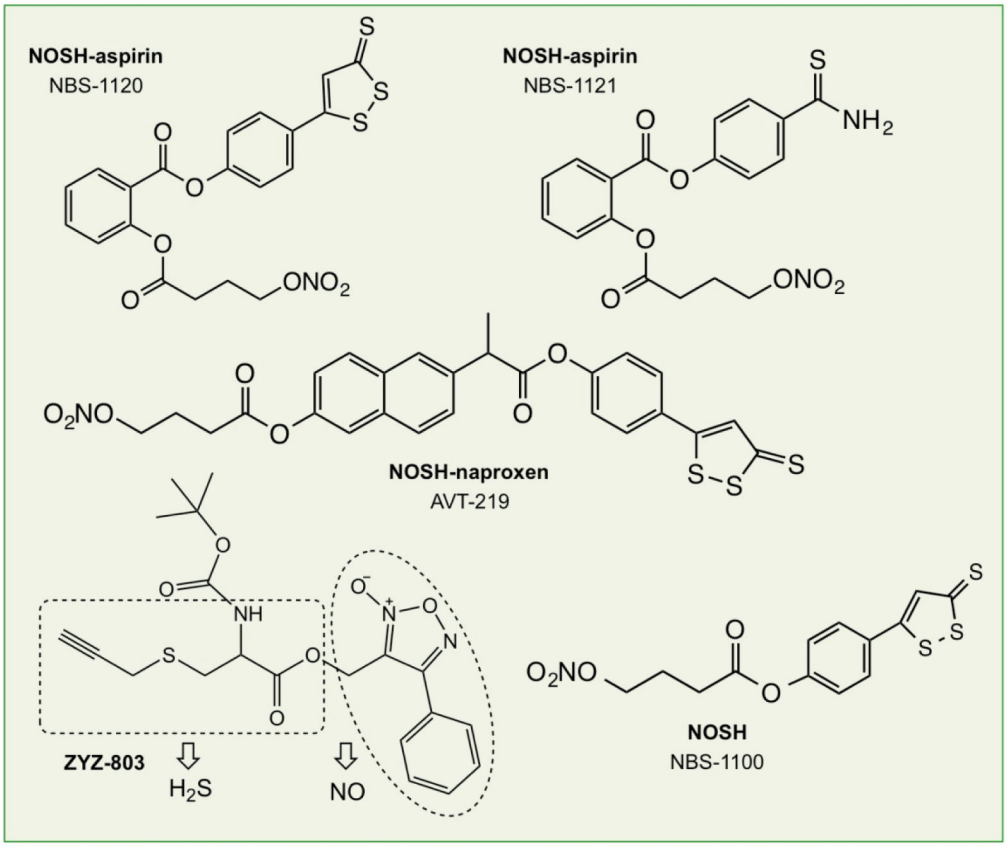

Figure 13.
Structural components of NOSH-aspirin (NBS-1120 and NBS-1121), NOSH-naproxen (AVT-219), NOSH (NBS1100), and ZYZ-803.