NOVEL TOOLS FOR THE ELUCIDATION
OF PHYSIO-PATHOLOGICAL ROLE OF H₂S

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1. Introduction

Hydrogen sulfide is a chemical compound, made up of two hydrogens and one sulfide atom, hence the chemical formula H$_2$S. It is a colorless, flammable, water-soluble gas with the odor of rotten eggs.

The initial observations by Kimura’s group suggesting hydrogen sulfide is a biologically relevant signaling molecule have been followed by a myriad of studies demonstrating some effect of this gas on virtually every organ system and tissue. Growing evidence has shown that in addition to nitric oxide (NO) and carbon monoxide (CO), H$_2$S is a third gasotransmitter (Wang, 2002).

Hydrogen sulfide is now recognized as a ubiquitous gaseous signaling molecule that plays important and different roles in the endocrine, neuronal and cardiovascular system (Szabo, 2007).

1.1. H$_2$S chemistry and biology

Hydrogen sulfide is a gas with a structure very similar to that of water, but this is where the similarity ends (Figure 1). The sulfur atom is not nearly as electronegative as oxygen so that hydrogen sulfide is much less polar than water.

![Figure 1](image)

*Figure 1* - Similarity in molecular structure between water (H$_2$O) and hydrogen sulfide (H$_2$S)
Because of this, comparatively weak intermolecular forces exist for H$_2$S and the melting and boiling points are much lower than they are in water. The boiling temperatures of hydrogen sulfide and water are -60.7 and 100.0 °C, respectively (Caliedo, 2010).

Hydrogen sulfide is weakly acidic, dissociating in aqueous solution into hydronium cation (H$_3$O$^+$) and hydrosulfide anion (HS$^-$), which subsequently may decompose to H$_3$O$^+$ and sulfide ion (S$^{2-}$) ($K_{a1}=1.3 \times 10^{-7}$M, $K_{a2}=1 \times 10^{-19}$M).

$$
\begin{align*}
\text{H}_2\text{S} & \rightleftharpoons \text{H}_2\text{O} + \text{HS}^- \\
\text{HS}^- & \rightleftharpoons \text{H}_2\text{O} + \text{S}^{2-}
\end{align*}
$$

Figure 2- Chemical equilibrium of H$_2$S in water

Under physiological conditions, i.e., at pH 7.4, one-third of hydrogen sulfide is undissociated and present in biological fluids as H$_2$S.

1.2. H$_2$S biosynthesis and metabolism

In mammalian systems, H$_2$S may be produced by two pyridoxal-5'-phosphate (PLP)-dependent enzymes (vitamin B$_6$), cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE aka CGL, EC 4.4.1.1), as well as cysteine aminotransferase (CAT, EC 2.6.1.3) and 3-mercaptopyruvate sulfurtransferase (3-MST, EC 2.8.1.2).

These enzymes are involved in transsulfuration and reverse transsulfuration pathways in different capacities and utilize specific substrates (Figure 3).
Figure 3 - Biosynthesis and transformation of $\text{H}_2\text{S}$ in mammalian cells. CBS, cystathionine $\beta$-synthase; CSE, cystathionine $\gamma$-lyase; CAT, cysteine aminotransferase; MST, 3-mercaptopyruvate sulfurtransferase.

1.2.1. Enzymatic Production of $\text{H}_2\text{S}$

Initially CBS and CSE catalysed reactions were thought to be the primary pathways for $\text{H}_2\text{S}$ production. CBS catalyzes the $\beta$-replacement reaction of homocysteine with serine to form cystathionine, thereby irreversibly committing this reaction into the transsulfuration pathway (Stipanuk, 2004).

CSE then catalyzes the $\alpha,\gamma$-elimination of cystathionine to form cysteine, $\alpha$-ketobutyrate and $\text{NH}_3$. Both CBS and CSE can then generate $\text{H}_2\text{S}$ from cysteine via $\beta$-elimination reactions (Singh, 2009).

A combination of homocysteine and cysteine constitutes the optimal substrate for CBS, generating cystathionine and $\text{H}_2\text{S}$ (Figure 3).
Furthermore, CSE is capable of using L-cysteine as the substrate to form two gases, H₂S and NH₃ and pyruvate. The involvement of CSE in other elimination reactions includes the catalysis of L-homoserine to form H₂O, NH₃, and 2-oxobutanoate and that of L-cystine to form thiocysteine, pyruvate, and NH₃.

CAT transfers the amine group from cysteine to a keto acid, typically α-ketoglutarate, forming 3-mercaptopyruvate. Subsequent desulfuration of 3-mercaptopyruvate by 3-MST forms the persulfide, 3-MST-SSH. MST would then transfer the sulfur from 3-mercaptopyruvate to sulfite or other sulfur acceptors or form elemental sulfur. The direct outcome of the CAT-MST pathway is the production of sulfane sulfur (or bound sulfur), not the free form of H₂S. H₂S would be consequently formed either through reduction of the atomic sulfur or released from thiosulfate or persulfides.

The former requires the presence of reductants (Kabil, 2010) and the latter, specific enzymes such as thiosulfate sulphur transferase or thiosulfate reductase (Stipanuk, 1982).

CBS and CSE are cytosolic enzymes. CBS was originally considered to be the predominant enzyme for H₂S production in the brain, whereas H₂S synthesis in the heart and vasculature was attributed to CSE (Kimura, 2011).

Recent studies with improved markers have provided a broader picture of enzyme distribution, e.g., CBS in vascular endothelium, CAT and 3-MST in vascular endothelium and brain and MST, but not CAT, in vascular smooth muscle (Olson, 2010). CAT and 3-MST have been found in both mitochondria and cytosol (Kamoun, 2004), although 3-MST may be more localized to the mitochondrial matrix (Mikami, 2011).
The most commonly used agents to inhibit H$_2$S biosynthesis include propargylglycine (PGG), β-cyanoalanine (BCA), aminooxyacetic acid (AOAA), trifluoroalanine and hydroxylamine (HA) (Szabo, 2007; Whiteman et al., 2011). PGG and BCA are claimed to be specific inhibitors of CSE, while AOAA is often used as a selective CBS inhibitor.

1.2.2. Non-enzymatic production of H$_2$S

A minor endogenous source of H$_2$S is the non-enzymatic reduction of elemental sulfur to H$_2$S using reducing equivalents obtained from the oxidation of glucose in erythrocytes (Searcy, 1998). Human erythrocytes produce H$_2$S when provided with elemental sulfur or inorganic polysulfides.

Sulfide, via non-enzymatic oxidation, yields thiosulfate. The latter can be converted to sulfite by thiosulfate reductase in liver, kidney, or brain tissues or by thiosulfate sulfurtransferase in the liver. H$_2$S can also be released from thiosulfate and persulfides. Garlic and garlic-derived organic polysulfides induce H$_2$S production in a thiol-dependent manner (Benavides, 2007).

Instead, while garlic has long been felt beneficial as an antioxidant, recent evidence suggests that a number of beneficial effects of garlic are derived from H$_2$S production.

Thus far the best characterized naturally occurring H$_2$S-donating compound from garlic (*Allium sativum*) is allicin (diallylthiosulfinate) which decomposes in water to a number of compounds, such as diallyldisulfide (DADS) and diallyltrisulfide (DATS).
1.3. Key enzymes responsible for $\text{H}_2\text{S}$ production

1.3.1. Cystathionine beta-synthase

The human enzyme comprises 551 amino acids with a subunit molecular weight of \(~63\) kDa (Kraus, 1986). It is a tetramer, which is prone to aggregation, and binds one heme and one PLP per subunit. AdoMet functions as a V-type allosteric activator, binds stoichiometrically to each subunit (Taoka, 1999), and increases enzyme activity \(~2\)- to 3-fold (Figure 4).

The catalytic core represents the conserved part of the protein and resembles other members of the $\beta$- or fold II class of PLP-dependent enzymes (Alexander, 1994). The presence of protoporphyrin IX in cystathionine $\beta$-synthase is unique in the family of PLP-dependent enzymes and is seen in only a subset of organisms.

A glycine rich loop, G256–T257–G258–G259–T260, another conserved structural feature of fold II enzymes, makes multiple electrostatic interactions with the phosphate moiety of PLP (Figure 5). Based on the chemical shift of the enzyme-bound PLP in yeast and human cystathionine$\beta$-synthase, the phosphate appears to be bound as a dianion (Kabil, 2001).
Figure 4 - Crystal structure of dimeric human cystathionine β-synthase lacking the C-terminal regulatory. The figure was generated using the PDB file 1M54. The heme is shown in red and the PLP in yellow.

Figure 5 - Close up of the active site of human cystathionine β-synthase showing the conserved residues that interact with the PLP.
The human cystathionine β-synthase catalyzes the condensation of serine and homocysteine to give cystathionine and H₂O. Furthermore, the β-elimination reaction catalyzed by cystathionine-β-synthase has the potential for generating also H₂S, using homocysteine and cysteine as substrates (Figure 6).

![Figure 6 - Reactions catalysed by CBS.](image)

1.3.2. Cystathionine gamma-lyase

The three-dimensional structures of human and yeast CSE have recently been elucidated via X-ray crystallography (Sun, 2009).

Structurally, the enzyme consists of four identical monomers of approximately 45 kDa, with a covalently bound pyridoxal 5′-phosphate (PLP) cofactor in each monomer (Figure 7). However, some studies show evidence for differential PLP binding affinities among the monomers of CSE and transient dissociation or removal of PLP from the enzyme during the catalytic process (Messerschmidt, 2003).
Figure 7 - Crystal structure of human cystathionine γ-lyase. The figure was generated using the PDB file 2NMP. The PLP is shown in pink.

Based on the crystal structures of yeast and human CSE as well as on sequence alignment with other transsulfuration enzymes, several active-site residues that could be involved in catalyzing the α,β-elimination reaction, leading to H$_2$S production, were identified (Huang, 2010).

In the crystal structures of yeast and human CSE, several residues were found to contribute towards the binding of the PLP cofactor. These residues include Tyr60 and Arg62 from the adjacent monomer; Tyr114, which π-stacks with the pyridoxal ring of the cofactor; Asp187, which hydrogen bonds to the pyridoxal nitrogen; and Ser209 and Thr211, which provide hydrogen-bond contacts to the phosphate group. Studies have also shown that Lys212 serves as an important catalytic residue by forming a covalent bond to the PLP cofactor and
facilitating proton transfer reactions during the α,γ-elimination reaction of L-
cystathionine.

The location of the residues (only side chains shown) studied by site-
directed mutagenesis is shown in Figure 8, and significant hydrogen bonds and
polar interactions are depicted by dotted lines. Besides interacting with residues
from the same subunit, the PLP cofactor is hydrogen bonded to Tyr60* and Arg62*
from the adjacent subunit as well. The figure was produced by the program
PyMOL (DeLano, 2002).

Figure 8- Active site of the human CSE enzyme.

CSE is an enzyme that is found predominantly in mammals and some fungi
and is traditionally known for its role in the reverse transsulfuration pathway,
where L-methionine is converted into L-cysteine through a series of metabolic
interconversions.
Specifically, the role of CSE in this reaction pathway is to convert L-cystathionine into L-cysteine whilst generating α-ketobutyrate and ammonia (Figure 9). The reaction proceeds via an α,γ-elimination mechanism where the C–γ–S bond of L-cystathionine is specifically cleaved to yield L-cysteine.

Besides its role in the conversion of L-cystathionine into L-cysteine, studies have also shown that CSE can utilize L-cysteine as a substrate for producing H₂S via an α,β-elimination reaction (Figure 9).

### 1.4. Biological functions of hydrogen sulfide

Knowledge of the biological roles for endogenous H₂S is constantly expanding. Many studies in the literature indicate that H₂S executes physiological effects at a wide range of concentrations between 10 and 300 μM (Li, 2011).

It is clear that H₂S is involved in modulating various physiological responses including anti-inflammation (Zanardo, 2006), reducing oxidative stress (Yonezawa, 2007), neuromodulation (Abe, 1996), vasoregulation (Laggner, 2007), protection from reperfusion injury after myocardial infarction (Sivarajah, 2009), and inhibition of insulin resistance (Ali, 2007).
Moreover, many researchers continue to explore this signalling molecule for its involvement in various aspects of cell function, cytoprotection and cellular signalling.

1.4.1. Cardiovascular system

The growing knowledge about the biological significance of H\textsubscript{2}S in the heart and blood vessels is unravelling the importance of this gaseous mediator in the control of CV homeostasis.

In particular, it is emerging that H\textsubscript{2}S shares almost all the beneficial effects of NO, without being the source of toxic metabolites (Lefer, 2007). Recently, it has been proposed that the role of H\textsubscript{2}S in CV homeostasis becomes more important and critical, when the NO-mediated control is compromised (for example, in the case of endothelial dysfunction). H\textsubscript{2}S evokes relaxing responses in the vascular smooth muscle, and this action has been observed in large vessels, such as the rat thoracic aorta and portal vein, as well as (and with higher potency) in peripheral resistance vessels, which play a more significant role than large conduit arteries in the regulation of vascular resistance and blood pressure (Cheng, 2004). The vasomotor effects of H\textsubscript{2}S are mimicked by L-cysteine and the vasorelaxing activity of L-cysteine is abolished by the CSE inhibitor PGG, thus proving that L-cysteine acts as a H\textsubscript{2}S source. Consistently, genetic deletion of CSE in mice markedly reduces H\textsubscript{2}S levels in the serum, heart, and aorta, and mutant mice lacking CSE develop marked hypertension and a decreased endothelium-dependent vasorelaxant effect (Yang, 2008).
It is currently acknowledged that H$_2$S relaxes blood vessels mainly (not exclusively) by opening the K$_{ATP}$ channels of vascular smooth muscle cells. Together with vasorelaxing activity, H$_2$S (such as NO) is endowed with a wide range of additional biological roles, which are relevant for a polyedric control of the CV system. For example, H$_2$S inhibits platelet aggregation/adhesion induced by ADP, collagen, epinephrine, arachidonic acid, thromboxane mimetic U46619, and thrombin.

**1.4.2. Immune system and inflammation**

It is well known that nonsteroidal anti-inflammatory drugs (NSAIDs) induce gastroenteropathy (Fiorucci, 2005). Research suggests that NSAIDs suppressed endogenous H$_2$S synthesis by reducing expression of CSE.

The accompanying reduction of H$_2$S synthesis may in turn contribute to an increase in leukocyte adherence resulting in gastric injury that is seen after NSAID administration (Perini, 2004).

Similarly, administration of exogenous H$_2$S reduced the ability of these agents to cause gastric injury. Exogenously supplied H$_2$S suppressed NSAID-induced granulocyte infiltration, expression of endothelial and leukocyte adhesion molecules, and expression of tumor necrosis factor $\alpha$ (TNF$\alpha$) (Fiorucci, 2005). It was found that leukocyte adhesion to the vascular endothelium induced by aspirin injury was decreased after increasing H$_2$S bioavailability and that CSE inhibition with propargylglycine exacerbated aspirin mediated mucosal injury and inflammation. This study also observed that leukocyte expression of LFA-1 was suppressed by exogenous H$_2$S. Interestingly, this article also showed a molecular
aspect of H$_2$S induced anti-inflammatory effects, such that H$_2$S donors decreased aspirin-induced leukocyte adhesion through the activation of K$_{ATP}$ channels and inhibition of CSE activity that promotes leukocyte adhesion. Additional studies demonstrated that co-administration of an H$_2$S donor with an NSAID resulted in inhibition of NSAID-induced leukocyte adherence and reduction of the severity of gastric damage (Wallace, 2007).

1.4.3. Respiratory system

The major environmental exposure to H$_2$S in humans is through the respiratory tract. On the basis of the H$_2$S relaxing action on the vascular smooth muscle, the relaxant property of H$_2$S has been investigated also on rings of bronchial smooth muscle of two rodent species. This study showed that H$_2$S causes strong relaxation in the isolated bronchus rings from the mouse, but produces only slight relaxation in guinea pig rings.

H$_2$S content and CSE activity are significantly enhanced also in isolated rat lung submitted to ischemia/reperfusion injury, and a preventive perfusion with H$_2$S attenuates such an injury, reducing malondyaldehyde (MDA) production and stimulating superoxide dismutase and catalase activity (Fu, 2008).

Concerning the endogenous CSE/H$_2$S pathway, it seems to play a relevant role in pulmonary hypertension (PH). In particular, this pathway has been found to be down-regulated in hypoxic PH (HPH), resulting in a decreased endogenous H$_2$S production in rat lung tissues due to oxidative stress. In particular, both the gene expression of CSE and the activity of this enzyme were suppressed in lung tissues during HPH, but the exogenous supply of H$_2$S resulted in enhancing CSE
activity, upregulating CSE gene expression in lung tissue and lessening pulmonary vascular structure remodeling during HPH (Zhang, 2003). After the treatment with exogenous H$_2$S, HPH was attenuated, through a direct scavenging of oxidized glutathione (GSSG), an increased total antioxidant capacity (Wei, 2008) and the inhibitory effect of H$_2$S on pulmonary vascular inflammation, associated with a high I-B (inhibitor of NF-B) expression and a down-regulation of NF-B p65 expression (Jin, 2008).

Furthermore, The CSE/H$_2$S pathway seems to exert beneficial roles also in the two most important obstructive airway diseases, such as asthma and chronic obstructive pulmonary disease (COPD). In an ovalbumin-induced asthmatic rat model and in human asthmatic patients, the levels of endogenous H$_2$S, whose anti-inflammatory effects are related to the inhibition of iNOS activity and decrease with the progression of disease severity (Fan, 2009). Consistently, increased levels of serum H$_2$S in milder COPD may play a useful role in airway protection, antagonizing oxidative stress and airway inflammation and preventing the progress of COPD.

1.4.4. Reproductive system

Recent studies focused on the effects of exogenous H$_2$S on mammalian reproductive systems. The powerful vasorelaxant effect of H$_2$S on the penile artery suggests an important physiological role of H$_2$S in the erectile response of human corpus cavernosum (Di Villa Bianca, 2009).

Specifically, NaHS (0.1–1 mM) relaxed the preconstricted human corpus cavernosum strips in vitro, independent of the presence of endothelium or the
function of endothelial NOS (eNOS). The relaxant effect of NaHS depends on the nature of the stimuli that were used to preconstrict the tissue strips.

1.4.5. Central Nervous System

As an almost ubiquitous bioactive molecule, H$_2$S exerts important regulatory effects in several biological systems. For instance, it is pivotally involved in the control of important functions in the CNS. A high expression of CBS in the rat hippocampus and cerebellum was observed (Abe, 1996).

In CNS neurons, H$_2$S enhances cAMP production, thus leading to an increased sensitivity of NMDA receptors to glutamate (Kimura, 2000).

This sensitization of NMDA receptors, elicited by quite high concentrations of H$_2$S (50–160 µM), contributes to the induction of hippocampal long-term potentiation, a process of synaptic plasticity involved in the mechanisms of learning and memory (Kimura, 2005). Increased production of cAMP activates protein kinase A which regulates brain function through intracellular protein phosphorylation, but this is not the only intracellular signalling in which H$_2$S seems to be involved.

In fact, H$_2$S may enhance reducing activity and protect neurons against oxidative stress via activation of upstream receptor tyrosine kinase (Tan, 2010).

Finally, recent findings reported that H$_2$S inhibits lipopolysaccharide(LPS)-induced NO production in microglia via inhibition of p38-MAPK (Hu, 2010) and that MAPKs regulate cellular activities, such as apoptosis, differentiation, metabolism, etc (Roux, 2004). These data suggest a role for H$_2$S in the treatment of cerebral ischemia and neuro-inflammatory diseases.
2. Aim of project

Retrospective analysis of the literature on biological effects of H$_2$S suggests two approaches for studying physio-pathological conditions in which it is involved and for the developing therapies based on its activities:

• use of H$_2$S in the gaseous state, or "sulfur-precursors" or "prodrugs" capable of releasing, directly or via interaction with specific enzymes, hydrogen sulfide;

• use of CBS/CSE inhibitors, leading to blockade of endogenous production of H$_2$S.

The aim of my PhD project is design, synthesis and structural characterization of three different series of compounds:

1. CBS/CSE substrates a/o inhibitors, crucial to study the pathophysiological implications of H$_2$S;

2. hydrogen sulfide-releasing hybrids, containing pharmacologically active compounds and molecules able to release H$_2$S, that could enhance therapeutic activity;

3. novel compounds able to release H$_2$S, non enzimatically, with slow kinetic of release, mimicking the physiological conditions.

Because of the unavailability of a pharmacophoric model as a lead for rational design of targeted enzyme substrates and inhibitors we preliminarily selected and tested commercially available cysteine surrogates. The catalytic profiles of recombinant CBS and CSE were assessed in the presence of the
following selected compounds: L-(S-carbamoyl)-cysteine; L-2-oxothiazolidin-4-carboxylic acid; L-3-(2-aminoethylthio)-2-aminopropanoic acid; L-thiazolidine-4-carboxylic acid; D,L-penicillamine.

On the basis of the obtained results, new compounds were designed; they were characterized by the introduction of a propargyl group in α-carboxyl position, aiming to obtain novel molecular entities embodying the structural features of both cysteine and the well known CSE inhibitor, DL-propargylglycine (PGG). In order to consider the small size of the active site channel, containing several patches of hydrophobic residues, we decided to introduce a butyl group in α-carboxyl position, to facilitate the access to the enzyme active site.

The structural modifications were also extended to aromatic scaffolds, to weaken π-stacking interaction between PLP and Tyr114, two important components of the active site (Table 1).

Further development of my PhD project is related to new classes of hydrogen sulfide-releasing moieties combined with pharmacologically active compounds. In particular, my attention has been paid to the synthesis of hydrogen sulfide-releasing hybrids, such as glucocorticoids-H₂S donors.

With regard to glucocorticoids, the choice is derived from the analysis of a recent in vivo study that investigated the effects of a H₂S donor (NaHS) on airways in balb/c mice sensitized with ovalbumin (OVA) (Roviezzo et al., submitted). In this study, aerosolized NaHS blunted OVA-induced airway hyperresponsiveness: these data suggest that combined early treatment with H₂S donors in association with glucocorticoids might improve asthma symptoms by inhibiting classical inflammatory signals and by acting, at the same time, on
airway myofibroblasts, perhaps delaying the onset of sub-epithelial fibrosis and the secretion of inflammatory factors and contributing to bronchodilation.

Considering the vast potential of hydrogen sulfide in asthma therapy, we have supposed that combined treatment with glucocorticoids and H$_2$S-releasing moieties could represent an effective therapeutic strategy for asthma care.

Starting from this consideration, glucocorticoids-H$_2$S donors have been designed: two most commonly used corticosteroids, dexamethasone and beclomethasone, were condensed with different H$_2$S-releasing compounds, such as 5-(p-hydroxyphenyl)-1,2-dithione-3-thione (ADT-OH) and 4-hydroxythiobenzamide (TBZ) (Table 2).

Concerning the third class of compounds, H$_2$S-releasing capacity is a pharmacologically relevant feature, already attributed to naturally occurring compounds, such as the diallyl polysulfides of garlic and our attention was thus devoted to the synthesis of ureic derivatives, as well as 1,2,4-thiadiazolidine-3,5-diones, representing a potential innovative scaffold for the release of H$_2$S (Table 3). These derivatives, in analogy with the N-(benzoylthio)benzamide compounds, recently reported in the literature, were evaluated according to their ability to release H$_2$S following the cleavage of the NH-S bond.
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3. Materials and methods

3.1. Chemistry

The synthetic route for the preparation of propargylamide (Ia-IIIa) and butylamide (Ib-IIIb) derivatives is reported in Scheme 1. The carboxylic acid was treated with N-(9-fluorenylmethoxycarbonyloxy) succinimide to protect the amine group, if needed. The coupling reaction of N-protected carboxylic acid with opportune amine, propargylamine hydrochloride or butylamine, was performed in the presence of TBTU, HOBt and N,N-diisopropylethylamine (DIPEA), leading to the formation of an amide bond. Fmoc deprotection, when necessary, was carried out using a solution of 33% DIEA in tetrahydrofuran, obtaining compounds Ia-IIIa and Ib-IIIb, respectively.

The general strategy for the synthesis of compounds IVa-IVb is summarized in Scheme 2, as follows: protection of amine and carboxyl groups; ether formation using the conventional Williamson synthesis, by treatment with a strong base, NaH or Na₂CO₃, and propargyl bromide or butyl bromide, respectively, performed in anhydrous CH₃CN; subsequent Boc deprotection and methyl ester hydrolysis, providing the final compounds IVa and IVb.

Scheme 3 depicts the synthetic pathways employed to prepare glucocorticoids-H₂S donors. Glucocorticoids, dexamethasone or beclomethasone, were converted to the corresponding 21-succinate by treatment with succinic anhydride in the presence of catalytic amount of DMAP in anhydrous pyridine. Coupling reaction of succinate derivative with H₂S releasing chemical moieties, such as ADT-OH or 4-hydroxythiobenzamide (TBZ), was performed in the
presence of EDC and DMAP, leading to the formation of glucocorticoids derivatives (FAS I-IV).

The synthetic strategy employed for the preparation of the 1,2,4-thiadiazolidin-3,5-diones is summarized in Scheme 4. The compounds (THIA I-VII) were synthesized through oxidative condensation of isothiocyanates with isocyanates in the presence of SO2Cl2 as the oxidizing agent.

**Scheme 1** - i) Na2CO3 9% /Fmoc-OSu/dioxane; ii) TBTU/HOBt/DIPEA; iii) 33% DIEA in THF.
Scheme 2- i) di-\textit{t}-butyl dicarbonate/triethylamine/H\textsubscript{2}O/dioxane; ii) DCC/MeOH; iii) potassium carbonate/acetonitrile; propargylamine hydrochloride; iv) sodium hydride/anhydrous acetonitrile; butylamine; vi) 1N sodium hydroxide solution/ethanol; vii) 40\% trifluoroacetic acid in dichloromethane solution.
Scheme 3- i) succinic anhydride/DMAP/anhydrous pyridine; ii) EDC/DMAP

Scheme 4- i) SOCl$_2$/diethyl ether; ii) H$_2$O, reflux
3.2. Experimental procedures

All reagents were commercial products purchased from Sigma Aldrich. Melting points, determined using a Buchi Melting Point B-540 instrument, are uncorrected and represent values obtained on recrystallized or chromatographically purified material. $^1$H-NMR spectra were recorded on Varian Mercury Plus 400 MHz instrument. Unless otherwise stated, all spectra were recorded in DMSO. Chemical shifts are reported in ppm using Me$_4$Si as internal standard. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), q (quartet), qt (quintet), dd (double doublet), m (multiplet). Mass spectra of the final products were performed on API 2000 Applied Biosystem mass spectrometer. Elemental analyses were carried out on a Carlo Erba model 1106; analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. All reactions were followed by TLC, carried out on Merck silica gel 60 F254 plates with fluorescent indicator and the plates were visualized with UV light (254 nm). Preparative chromatographic purifications were performed using silica gel column (Kieselgel 60). Solutions were dried over Na$_2$SO$_4$ and concentrated with a Buchi R-114 rotary evaporator at low pressure.

3.2.1. Cysteine surrogates: propargylamide and $n$-butylamide derivatives (Ia-IIIa and Ib-IIIb)

3.2.1.1. $3-$(((9H-fluoren-9-yl)methoxy)carbonyl)thiazolidine-4-carboxylic acid (Ia). The thiazolidine-4-carboxylic acid (1, 1g, 7.5mmol) in Na$_2$CO$_3$ 9% (10 mL) was cooled in ice water and mechanically stirred. Then a solution of Fmoc-OSu
(2.5g, 7.5mmol) and dioxane (20 mL) was added dropwise and the mixture was stirred for 2.5 hours at room temperature. The solvent was evaporated, the aqueous residue acidified with 1N HCl and the product extracted with ethyl acetate. The organic phase was then dried on anhydrous Na₂SO₄ and filtered, and the solvent was evaporated. The product was crystallized from diethyl ether, yielding 2.5 g of 3-((9H-fluoren-9-yl)methoxy)carbonyl)thiazolidine-4-carboxylic acid (1a, 94%) as a solid.

3.2.1.2. N-(prop-2-yln-1-yl)thiazolidine-4-carboxamide and N-butylthiazolidine-4-carboxamide (Ia and Ib). The intermediate 3-((9H-fluoren-9-yl)methoxy)carbonyl)thiazolidine-4-carboxylic acid (1a, 1g, 2.8mmol) was dissolved in DMF (30mL) and coupled to the opportune amine, propargylamine hydrochloride (0.258g, 2.8mmol) or butylamine (0.205g, 2.8mmol), in the presence of DIPEA (0.536mL, 3.1mmol), using HOBT (0.472g, 3.1mmol) and TBTU (0.988g, 3.1mmol) as the coupling reagents. This reaction was carried out at room temperature with stirring overnight. The solvent was evaporated, and the crude material was then dissolved in ethyl acetate (150mL) and washed with 5% citric acid (3 x 50mL), 10% NaHCO₃ (3 x 50mL) and brine (50mL). The organic layer was dried on anhydrous Na₂SO₄ and filtered, and the solvent was evaporated. After chromatography on a silica gel column (eluent, 7:3 ethyl acetate/hexane) 0.86 g and 0.82 g of intermediates I were obtained (82% for the propargylamine derivative and 75% for the n-butylamide derivative, respectively) as solids.
Fmoc deprotection of intermediates \textbf{I} was performed using a 33\% diethylamine solution in tetrahydrofuran and the mixture was stirred for 2.5 hours at room temperature. Afterward the solvent was concentrated in vacuo and the obtained residue was purified by column chromatography (dichloromethane/methanol 9.5:0.5 (v/v)). The combined and evaporated product fractions were crystallized from hexane, to give the final compounds N-(prop-2-yn-1-yl)thiazolidine-4-carboxamide \textbf{Ia} and N-butylthiazolidine-4-carboxamide \textbf{Ib} as white solids (obtained yields 72\% and 70\% respectively).

\textit{N-(prop-2-yn-1-yl)thiazolidine-4-carboxamide (Ia)} mp 126–127 °C; \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) \(\delta\) 3.13 (s, 1H, C\textsubscript{CH}), 3.10-3.18 (dd, 1H, CH\textsubscript{2}CH thiaz, J=6.65), 3.39-3.43 (dd, 1H, CH\textsubscript{2}CH thiaz, J=6.65), 3.88 (s, 2H, NHCH\textsubscript{2}), 3.96 (d, 1H, SCH\textsubscript{2}NH, J=9.90), 4.13 (t, 1H, CH\textsubscript{2}CH thiaz), 4.24 (d, 1H, SCH\textsubscript{2}NH, J=9.90), 8.29 (s, 1H, NH), 8.99 (s, 1H, NH); \textsuperscript{13}C NMR (400 MHz, DMSO-d\textsubscript{6}) \(\delta\) 29.0, 32.9, 61.8, 74.0, 74.6, 81.3, 170.1; ESI-HRMS (M + H)\textsuperscript{+} m/z calcd 170.23 for C\textsubscript{7}H\textsubscript{10}N\textsubscript{2}OS; found 171.

\textit{N-butylthiazolidine-4-carboxamide (Ib)} mp 126–127 °C; \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) \(\delta\) 0.91 (t, 3H, CH\textsubscript{3}, J=6.96, J=7.33), 1.30-1.36 (m, 2H, CH\textsubscript{2}CH\textsubscript{3}), 1.46-1.51 (qt, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}, J=6.65, J=7.04, J=7.43, J=7.04), 3.10-3.18 (dd, 1H, CH\textsubscript{2}CH thiaz, J=6.65), 3.25 (t, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}CH), 3.39-3.43 (dd, 1H, CH\textsubscript{2}CH thiaz, J=6.65), 3.96 (d, 1H, SCH\textsubscript{2}NH, J=9.90), 4.13 (t, 1H, CH\textsubscript{2}CH thiaz), 4.24 (d, 1H, SCH\textsubscript{2}NH, J=9.90), 8.29 (s, 1H, NH), 8.99 (s, 1H, NH); \textsuperscript{13}C NMR (400 MHz, DMSO-d\textsubscript{6}) \(\delta\) 14.1, 19.9, 31.5, 32.8, 38.9, 61.8, 74.6, 170.1; ESI-HRMS (M + H)\textsuperscript{+} m/z calcd 188.29 for C\textsubscript{8}H\textsubscript{16}N\textsubscript{2}OS; found 189.
3.2.1.3. 2-oxo-N-(prop-2-yn-1-yl)thiazolidine-4-carboxamide and N-butyl-2-oxothiazolidine-4-carboxamide (IIa and IIb). A mixture of 2-oxo-N-thiazolidine-4-carboxilic acid 2 (1g, 6.8 mmol), the opportune amine, propargylamine hydrochloride (0.622g, 6.8mmol) or butylamine (0.497g, 6.8mmol), HOBt (1.15g, 7.5mmol), TBTU (2.40g, 7.5mmol) and DIPEA (1.30mL, 7.5mmol) in DMF (20mL) was stirred overnight at room temperature. The solvent was concentrated to dryness and the crude mixture was purified by silica gel column chromatography using ethyl acetate/hexane 7:3 (v/v) as eluent. The crude product was recrystallized from hexane, yielding 0.96 g of 2-oxo-N-(prop-2-yn-1-yl)thiazolidine-4-carboxamide IIa (76%) and 0.94 g of N-butyl-2-oxothiazolidine-4-carboxamide IIb (69%), respectively.

2-oxo-N-(prop-2-yn-1-yl)thiazolidine-4-carboxamide (IIa) mp 131.5–132.4 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 3.13 (s, 1H, C\(\equiv\)CH), 3.31 (t, 2H, CH\(_2\)), 3.62-3.68 (dd, 2H, CH\(_2\) thiaz, J=8.61), 3.88 (s, 2H, NHCH\(_2\)), 4.27 (t, 1H, CH\(_2\)CH thiaz), 8.27 (s, 1H, NH), 8.53 (s, 1H, NH); \(^{13}\)C NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 29.0, 32.9, 56.8, 74.0, 81.3, 170.6, 174.0; ESI-HRMS (M + H)\(^+\) m/z calcd 184.22 for C\(_7\)H\(_8\)N\(_2\)O\(_2\)S; found 184.9.

N-butyl-2-oxothiazolidine-4-carboxamide (IIb) mp 83.2-83.7 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 0.85 (t, 3H, CH\(_3\)), J=7.04, J=7.43), 1.21-1.30 (m, 2H, CH\(_2\)CH\(_3\)), 1.34-1.41 (qt, 2H, CH\(_2\)CH\(_2\)CH\(_3\)), J=6.65, J=7.04, J=7.43, J= 7.04), 3.04-3.09 (dd, 1H, CH\(_2\)CH thiaz, J=6.65), 3.60-3.65 (dd, 1H, CH\(_2\)CH thiaz, J= 8.61), 4.22 (t, 1H, CH\(_2\)CH thiaz), 7.99 (s, 1H, NH), 8.23 (s, 1H, NH); \(^{13}\)C NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 29.0, 32.9, 56.8, 74.0, 81.3, 170.6, 174.0; ESI-HRMS (M + H)\(^+\) m/z calcd 218.28 for C\(_9\)H\(_{12}\)N\(_2\)O\(_2\)S; found 218.9.
MHz, DMSO-d$_6$ $\delta$ 14.1, 19.9, 31.5, 32.8, 38.9, 56.9, 170.1, 173.7; ESI-HRMS (M + H)$^+$ m/z calcd 202.27 for C$_8$H$_{14}$N$_2$O$_2$S; found 203.0.

3.2.1.4. 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-(thiophen-2-yl)propanoic acid (3a). The 2-amino-3-(thiophen-2-yl)propanoic acid (3, 1g, 5.8mmol) in Na$_2$CO$_3$ 9% (10mL) was cooled in ice water and mechanically stirred. Then a solution of Fmoc-OSu (1.97g, 5.8mmol) and dioxane (20mL) was added dropwise and the mixture was stirred for 2.5 hours at room temperature. The solvent was evaporated, the aqueous residue acidified with 1N HCl and the product extracted with ethyl acetate. The organic phase was then dried on anhydrous Na$_2$SO$_4$ and filtered, and the solvent was evaporated. The product was crystallized from diethyl ether, yielding 2.08 g of 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-(thiophen-2-yl)propanoic acid (3a; 91%) as a solid.

3.2.1.5. 2-amino-N-(prop-2-yn-1-yl)-3-(thiophen-2-yl)propanamide and 2-amino-N-butyl-3-(thiophen-2-yl)propanamide (IIIa and IIIb). The intermediate 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-(thiophen-2-yl)propanoic acid (3a, 1.0g, 2.5mmol) was dissolved in DMF (30mL) and coupled to the opportune amine, propargylamine hydrochloride (0.229g, 2.5mmol) or butylamine (0.183g, 2.5mmol), in the presence of DIPEA (0.48mL, 2.8mmol), using HOBt (0.42g, 2.8mmol) and TBTU (0.885g, 2.8mmol) as the coupling reagents. This reaction was carried out at room temperature with stirring overnight. The solvent was evaporated, and the crude material was then dissolved in ethyl acetate (150 mL) and washed with citric acid 5% (3 x 50 mL), NaHCO$_3$ 10% (3 x 50 mL) and brine (50 mL). The organic layer was dried on anhydrous Na$_2$SO$_4$ and filtered, and the
solvent was evaporated. After chromatography on a silica gel column (eluent, 7:3 ethyl acetate/hexane) 0.90 g and 0.98 g of intermediates III were obtained (76% for the propargylamide derivative and 87% for the n-butylamide derivative, respectively) as solids. A solution of 33% diethylamine solution in tetrahydrofuran was used during deprotection of Fmoc group of intermediates III. The mixture was stirred for 2.5 hours at room temperature. Afterward the solvent was concentrated in vacuo and the obtained residue was purified by column chromatography (dichloromethane/methanol 9.5:0.5 (v/v)). The combined and evaporated product fractions were crystallized from hexane, to give the final compounds N-(prop-2-yn-1-yl)tiazolidin-4-carboxamide IIIa and 2-aminon-butyl-3-(thiophen-2-yl)propanamide IIIb as white solids (yield: 68% and 75%, respectively).

2-amino-N-(prop-2-yn-1-yl)-3-(thiophen-2-yl)propanamide (IIIa). mp 196.2-196.3°C; ¹H NMR (400 MHz, DMSO-d₆) δ 3.18 (s, 1H), 3.27-3.31 (dd, 2H, CH₂CH, J=6.06), 3.90 (s, 2H, NHCH₂), 3.95 (t, 1H, CHCO, J=6.09), 6.94-6.97 (m, 2H, H-thioph), 7.40 (d, 1H, H-thioph, J=3.9), 8.38 (s, 2H, NH), 9.05 (s, 1H, NH₂); ¹³C NMR (400 MHz, DMSO-d₆) δ 28.9, 31.4, 53.9, 74.6, 80.7, 126.4, 127.9, 128.4, 136.4, 168.0; ESI-HRMS (M + H)⁺ m/z calcd 208.28 for C₇H₁₂N₂OS; found 209.2.

2-amino-N-butyl-3-(thiophen-2-yl)propanamide (IIIb). mp 172.9-173.3°C; ¹H NMR (400 MHz, DMSO-d₆) δ 0.85 (t, 3H, CH₃, J=7.43), 1.21-1.30 (m, 2H, CH₂CH₃), 1.34-1.41 (qt, 2H, CH₂CH₂CH₃, J=6.65, J=7.04, J=7.43, J= 7.04), 3.20 (d, 2H, NHCH₂ J= 6.03), 3.27-3.31 (dd, 2H, CH₂CH, J=6.06),3.95 (t, 1H,
CHCO, J=6.09), 6.94-6.97 (m, 2H, H-thioph), 7.40 (d, 1H, H-thioph, J=3.9), 8.38 (s, 2H, NH), 9.05 (s, 1H, NH₂); ¹³C NMR (400 MHz, DMSO-d₆) δ 14.1, 19.9, 31.4, 38.9, 53.9, 56.9, 126.4, 127.9, 128.4, 136.4, 168.0; ESI-HRMS (M + H)⁺ m/z calcd 226.34 for C₁₁H₁₈N₂OS; found 227.0.

3.2.1.6. Methyl 5-(tert-butoxycarbonylamino)-2-hydroxybenzoate (6). To a solution of 5-amino-salicylic acid (4, 2g, 13.0mmol) in 25 ml of dioxane and 12.5 mL of water, triethylamine (2.6mL, 19.5mmol) and di-tert-butyl-dicarbonate (4.2g, 19.5mmol) were added with stirring at 0° C for 1/2 h. The reaction mixture was stirred mechanically for 24 h at room temperature. After evaporation of the solvent, 3N HCl (15mL) was added dropwise to the residue. The precipitate is filtered, washed with water, dried and recrystallized from diethyl ether. 5-(tert-butoxycarbonylamino)-2-hydroxybenzoic acid was obtained (5) (3.28g, 100 % yield) and the powder (5, 3g, 11.8mmol) was dissolved in anhydrous methanol. To the solution, cooled to 0 °C, dicyclohexylcarbodiimide (2.4g, 11.8mmol) was added and the mixture was stirred at room temperature for 3h. The solvent was evaporated, the residue was taken up in ethyl acetate and dicyclohexylurea (DCU) was filtered. After chromatography on a silica gel column (eluent, 7:3 ethyl acetate/hexane) 2.3 g of 6 was obtained (73%) as a white solid.

3.2.1.7. 5-amino-2-(prop-2-ynyloxy)benzoic acid (IVA). Intermediate 6 (1g, 4.0mmol) in anhydrous acetonitrile was treated with potassium carbonate (0.475g, 4.0mmol) and then propargyl bromide (solution 80% wt. in toluene) (0.446mL, 4.0mmol) was added slowly into the flask. The reaction mixture was stirred under reflux overnight. After this period, carbonate was filtered and solvent concentrated
in vacuo. The residue was purified by column chromatography (3:7 ethyl acetate/hexane (v/v)). The combined and evaporated product fractions were crystallized from diethyl ether/hexane, affording 0.734g (62% yield) of methyl 5-(tert-butoxycarbonylamino)-2-(prop-2-ynyloxy)benzoate (7). To a solution of obtained compound (7, 0.734g, 2.5mmol) in ethanol, sodium hydroxide was added and the mixture was mechanically stirred at room temperature for 2 hours. The solvent was removed in vacuo and residue was slightly acidified with 1N hydrochloric acid. The aqueous solution was extracted with ethyl acetate and the organic phase was dried over anhydrous Na$_2$SO$_4$, filtered and evaporated to dryness to afford 0.734 g of compound 9 as a white powder (100% yield).

The crude intermediate (9, 0.734g, 2.5mmol) was treated with a solution of 40 % TFA in DCM. After 2 h the solvent was removed to obtain the crude product. The residue was loaded on a silica gel open column and eluted with ethyl acetate/hexane (7:3 v/v). The combined and evaporated fractions were crystallized from hexane to yield 0.386 g (87%) of pure product IVa. mp 220.0-220.4°C; $^1$H NMR (400 MHz, DMSO-$d_6$); $\delta$ 3.48 (s, 1H, CH), 4.63 (s, 2H, CH$_2$), 6.69 (d, 1H, J=8.43, Ar-H), 6.89 (d, 1H, J=8.43, Ar-H), 6.91 (s, 1H, Ar-H); $^{13}$C NMR (400 MHz, DMSO-$d_6$) $\delta$ 58.43 78.69, 80.50, 116.55, 118.35, 118.88,123.74, 143.42, 148.11, 168.04; ESI-HRMS (M + H)$^+$ m/z calcd 191.18 for C$_{10}$H$_9$NO$_3$; found 192.

3.2.1.8. 5-amino-2-butoxybenzoic acid (IVb). Intermediate 6 (1g, 4.0mmol) in anhydrous acetonitrile was treated with sodium hydride (0.097g, 4.0mmol) and then 1-butyl bromide (0.410 mL, 4.0mmol) was added slowly into the flask. The
reaction mixture was stirred under reflux overnight. The solvent was concentrated \textit{in vacuo} and the crude material was then dissolved in ethyl acetate (150 mL) and washed with citric acid 5% (3 x 50 mL), NaHCO$_3$ 10% (3 x 50 mL) and brine (50 mL). The organic layer was dried on anhydrous Na$_2$SO$_4$ and filtered, and the solvent was evaporated. The residue was purified by column chromatography (2:8 diethyl ether/hexane (v/v)). The combined and evaporated product fractions were crystallized from diethyl ether/hexane, affording 0.957 g (74% yield) of methyl 2-butoxy-5-(\textit{tert}-butoxycarbonylamino)benzoate (8).

To a solution of the obtained compound (8, 0.957g, 2.74mmol) in ethanol, sodium hydroxide was added and the mixture was mechanically stirred at room temperature for 2 hours. The solvent was removed \textit{in vacuo} and residue was slightly acidified with 1N hydrochloric acid. The aqueous solution was extracted with ethyl acetate and the organic phase was dried over anhydrous Na$_2$SO$_4$, filtered and evaporated to dryness to afford 0.908 g of 2-butoxy-5-(\textit{tert}-butoxycarbonylamino)benzoic acid (10) as a white powder (99% yield).

The crude intermediate (10, 0.908g, 2.9mmol) was treated with a solution of 40% TFA in DCM. After 2 h the solvent was removed to obtain the crude product. The residue was loaded on a silica gel open column and eluted with ethyl acetate/hexane (7:3 v/v). The combined and evaporated fractions were crystallized from hexane to yield 0.543 g (90%) of pure product \textbf{IVb}. mp 213.0-213.1°C; $^1$H NMR (400 MHz, DMSO-$d_6$); $\delta$ 0.89 (t, 3H, J=6.96, J=7.33, CH$_3$), 1.40-1.45 (m, 2H, CH$_2$CH$_3$), 1.65-1.68 (qt, 2H, CH$_2$CH$_2$CH$_3$), 4.0 (t, 2H, J= 5.86, J=6.23, OCH$_2$, 7.16 (d, 1H, J=9.16, Ar-H), 7.39 (d, 1H, J=8.43, Ar-H), 7.55 (s, 1H, Ar-H); $^{13}$C NMR (400 MHz, DMSO-$d_6$) $\delta$ 14.33, 19.28, 31.31, 69.143, 115.47, 122.96,
125.07, 127.45, 130.11, 156.84, 167.16; ESI-HRMS (M + H)$^+$ m/z calcd 209.24 for C$_{11}$H$_{15}$NO$_3$; found 210.

3.2.2. Synthesis of glucocorticoids-H$_2$S donors (FAS I-IV)

3.2.2.1. Dexamethasone succinate (I3). 1.0g (2.55mmol) of dexamethasone 11 was dissolved in anhydrous pyridine (30mL) and 0.77g (7.65mmol) of succinic anhydride and 0.1eq DMAP were then added. After being stirred overnight at room temperature, the mixture was evaporated under reduced pressure. The resulting residue was then treated with 20 mL of water and the mixture was stirred for 20 min and then centrifuged. The obtained precipitate was washed again with H2O and filtered. White powder of the product 13 (1.12g) was obtained in a yield of 89%. mp 188-189 °C

3.2.2.2. Dexamethasone-succinate-TBZ (FAS I). To a solution of 13 (1g, 2.0mmol) in anhydrous tetrahydrofuran (30mL), 4-hydroxythiobenzamide (0.613g, 4.0mmol) and DMAP (0.024g, 0.2mmol) were added. The reaction mixture was kept on ice bath stirring under nitrogen for 10' and EDC (0.575g, 3.0mmol) was then added. After the addition, the ice bath was removed and the mixture was stirred under nitrogen atmosphere for 3 hour. The solvent was evaporated and the residue was purified by column chromatography on silica (ethyl acetate: hexane, 6:4). The obtained product FAS I was recrystallized from hexane, yielding 0.98g (78 %) of product as a colorless solid. mp 144.5–146.1 °C

$^1$H NMR (DMSO-d$_6$) δ 0.78 (d, 3H, J=7.04), 0.88 (s, H), 1.05-1.10 (m, 1H), 1.48 (s, 3H), 1.53-1.79 (m, 4H), 1.88 (s, 3H), 2.09-2.18 (m, 1H), 2.26-2.40 (m,
2H), 2.58-2.61 (m, 3H), 2.79 (t, 2H, J=6.65, J=5.48), 2.82-2.91 (m, 1H), 2.87 (t, 2H, J=5.87, J=6.26), 5.16 (s, 2H), 5.99 (s, 1H), 6.21 (d, 1H, J=10.17), 7.14 (d, 1H, Ar-H, J=8.61), 7.28 (d, 1H, Ar-H, J=10.17), 7.91 (d, 2H, Ar-H, J=8.61), 9.50 (s, 1H, NH), 9.87 (s, 1H, NH); \(^{13}\)C NMR (DMSO-d\(_6\)) \(\delta\) 15.56, 16.70, 23.45, 27.96, 28.87, 29.36, 30.09, 32.35, 35.86, 36.13, 43.75, 48.21, 48.43, 68.70, 71.30, 90.94, 100.10, 121.61, 124.54, 127.18, 129.43, 137.52, 153.04, 153.10, 167.81, 170.92, 171.81, 185.73, 199.48, 205.11. ESI-HRMS (M + H)\(^+\) m/z calcd 627.72 for C\(_{35}\)H\(_{38}\)FNO\(_8\)S; found 628.0.

### 3.2.2.3. Dexamethasone-succinate-ADT (FAS II)

The desired product FAS II has been obtained following the same procedure above reported for FAS I starting from 13 (1g, 2.0mmol) and 5-(p-hydroxyphenyl)-1,2-dithione-3-thione (ADT-OH) (0.904g, 4.0mmol). ADT-OH was prepared from anethole and sulphur in dimethylformamide. Yield: 1.13g (81 %) of product FAS II as a colorless solid. mp 134.8–135.1 °C \(^1\)H NMR (DMSO-d\(_6\)) \(\delta\) 0.78 (d, 3H, J=7.04), 0.88 (s, H), 1.05-1.10 (m, 1H), 1.48 (s, 3H), 1.53-1.79 (m, 4H), 1.88 (s, 3H), 2.09-2.18 (m, 1H), 2.26-2.40 (m, 2H), 2.58-2.61 (m, 3H), 2.80 (t, 2H, J=6.65, J=5.48), 2.82-2.91 (m, 1H), 2.89 (t, 2H, J=5.87, J=6.26), 5.40 (s, 2H), 5.47 (d, 1H, J=10.17), 5.73 (s, 1H), 5.96 (s, 1H), 6.21 (d, 1H, J=10.17), 7.14 (d, 2H, Ar-H, J=8.61), 7.80 (s, 1H), 7.95 (d, 2H, Ar-H, J=8.61); \(^{13}\)C NMR (DMSO-d\(_6\)) \(\delta\) 15.57, 16.70, 23.40, 27.72, 28.88, 29.41, 30.71, 32.35, 34.13, 35.87, 36.12, 43.75, 48.27, 48.44, 68.71, 70.76, 71.12, 90.94, 123.49, 124.56, 129.12, 129.28, 129.45, 136.26, 153.18, 153.85, 167.50, 170.85, 171.83, 173.15, 185.72, 205.13. ESI-HRMS (M + H)\(^+\) m/z calcd 700.86 for C\(_{35}\)H\(_{37}\)FNO\(_8\)S\(_3\); found 700.
3.2.2.4. *Beclomethasone succinate (14).* The desired product 14 has been obtained following the same procedure previously reported for compound 13 starting from 1.0 g (2.45 mmol) of beclomethasone 12, 0.73 g (7.35 mmol) of succinic anhydride and 0.1 eq DMAP. Yield: 1.15 g (14, 92%). mp 180–182.5 °C

3.2.2.5. *Beclomethasone-succinate-TBZ (FAS III).* The desired product FAS III has been obtained following the same procedure above reported for FAS I starting from 14 (1g, 2.0 mmol) and 4-hydroxythiobenzamide (0.613g, 4.0 mmol). Yield: 0.87g (67 %) of FAS III as a colorless solid. mp 114.7–115.2 °C

1H NMR (DMSO-d$_6$) δ 0.92 (s, 3H), 1.01 (d, 3H, J=7.04), 1.05-1.10 (m, 1H), 1.48 (s, 3H), 1.53-1.79 (m, 4H), 1.88 (s, 3H), 2.09-2.18 (m, 1H), 2.26-2.40 (m, 2H), 2.58-2.61 (m, 3H), 2.79 (t, 2H, J=6.65, J=5.48), 2.82-2.91 (m, 1H), 2.87 (t, 2H, J=5.87, J=6.26), 5.16 (s, 2H), 5.99 (s, 1H), 6.21 (d, 1H, J=10.17), 7.14 (d, 2H, Ar-H, J=8.61), 7.28 (d, 1H, J=10.17), 7.91 (d, 2H, Ar-H, J=8.61), 9.50 (s, 1H, NH), 9.87 (s, 1H, NH);

13C NMR (DMSO-d$_6$) δ 17.66, 20.20, 24.87, 28.04, 28.96, 29.40, 30.38, 34.50, 35.93, 43.26, 47.32, 49.33, 50.17, 70.02, 74.54, 86.01, 88.32, 121.64, 124.45, 129.03, 129.17, 137.52, 153.07, 153.24, 167.22, 170.95, 171.79, 185.69, 199.49, 205.81. ESI-HRMS (M + H)$^+$ m/z calcd 644.17 for C$_{33}$H$_{38}$ClO$_8$S; found 643.8.

3.2.2.6. *Beclomethasone-succinate-ADT (FAS IV).* The desired product FAS IV has been obtained following the same procedure above reported for FAS I starting from 14 (1g, 2.0 mmol) and 5-(p-hydroxyphenyl)-1,2-dithione-3-thione (0.904g, 4.0 mmol). Yield: 1.1g (78 %) of FAS IV as a colorless solid.mp 127.7–128.5 °C

1H NMR (DMSO-d$_6$) δ0.92 (s, 3H), 1.01 (d, 3H, J=7.04), 1.05-1.10 (m, 1H),
1.48 (s, 3H), 1.53-1.79 (m, 4H), 1.88 (s, 3H), 2.09-2.18 (m, 1H), 2.26-2.40 (m, 2H), 2.58-2.61 (m, 3H), 2.80 (t, 2H, J=6.65, J=5.48), 2.82-2.91 (m, 1H), 2.89 (t, 2H, J=5.87, J=6.26), 5.40 (s, 2H), 5.47 (d, 1H, J=10.17), 5.73 (s, 1H), 5.96 (s, 1H), 6.21 (d, 1H, Ar-H, J=8.61), 7.14 (d, 2H, Ar-H, J=8.61), 7.80 (s, 1H), 7.95 (d, 2H, Ar-H, J=8.61); 13C NMR (DMSO-d6) δ 15.57, 16.70, 23.40, 27.72, 28.88, 29.41, 30.71, 32.35, 34.13, 35.87, 36.12, 43.75, 48.27, 48.44, 68.71, 70.76, 71.12, 90.94, 123.49, 124.56, 129.12, 129.28, 129.45, 136.26, 153.18, 153.85, 167.50, 170.85, 171.83, 173.15, 185.72, 205.13. ESI-HRMS (M + H)⁺ m/z calcd 717.3 for C35H37ClO8S3; found 716.8.

3.2.3. General procedure for the preparation of 1,2,4-thiadiazoline-3,5-diones (THIA I-VII)

A three-necked flask was equipped with a mechanical stirrer and charged with an ice-cold solution of corresponding isothiocyanate (1eq) and isocyanate (1eq) in anhydrous diethyl ether. Sulfuryl chloride (1eq), dissolved in a minimum volume of anhydrous diethyl ether, was added dropwise under inert atmosphere, keeping the mixture at 0 °C. After completion of the addition, the solution was stirred at room temperature for 48 h. The obtained solid was filtered, dissolved in 10 mL of water and vigorously stirred under reflux for 30 minutes. The reaction mixture was gradually cooled to 0 °C and the solid was recrystallized from water, filtered and dried to yield the pure product as a white solid.

3.2.3.1. 2,4-diphenyl-1,2,4-thiadiazoline-3,5-dione (THIA I). Compound THIA I was prepared from phenyl-isothiocyanate (1g, 7.4mmol) and phenyl-isocyanate (0.881g, 7.4mmol) according to a procedure similar to that described above.
Yield=74%: mp 119.1−120 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 7.31 (t, 2H, Ar-H, J=7.04, J=6.65), 7.46 (t, 4H, Ar-H, J=12.52, J=13.78), 7.55 (d, 2H, Ar-H, J=7.82), 7.58 (d, 2H, Ar-H, J=7.43); $^{13}$C NMR (400 MHz, CDCl$_3$) δ 123.5, 127.2, 127.4, 129.4, 129.5, 129.6, 132.6, 135.8, 150.4, 164.7; ESI-HRMS (M+H)$^+$ m/z calcd 270.31 for C$_{14}$H$_{10}$N$_2$O$_2$S; found 271.1.

3.2.3.2. 4-(4-methoxyphenyl)-2-phenyl-1,2,4-thiadiazoline-3,5-dione (THIA II)

Compound THIA II was prepared from phenyl-isothiocyanate (1g, 7.4mmol) and 4-methoxyphenyl-isocyanate (0.958mL, 7.4mmol) according to a procedure similar to that described above. Yield=72%: mp 155.3−155.4 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 3.83 (s, 3H, OCH$_3$), 6.95 (d, 2H, Ar-H, J=9.0), 7.43-7.47 (m, 5H, Ar-H), 7.52 (d, 2H, Ar-H, J=7.43); $^{13}$C NMR (400 MHz, CDCl$_3$) δ 55.6, 114.8, 126.3, 127.3, 128.1, 129.3, 129.4, 132.7, 150.8, 158.9, 165.0; ESI-HRMS (M+H)$^+$ m/z calcd 300.33 for C$_{15}$H$_{12}$N$_2$O$_3$S; found 301.1.

3.2.3.3. 4-(4-methoxyphenyl)-2-p-tolyl-1,2,4-thiadiazoline-3,5-dione (THIA III)

Compound THIA III was prepared from p-tolyl-isothiocyanate (1g, 6.7mmol) and 4-methoxyphenyl-isocyanate (0.870mL, 6.7mmol) according to a procedure similar to that described above. Yield=74%: mp 156.3−156.5 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 2.41 (s, 3H, -CH$_3$), 3.83 (s, 3H, -OCH$_3$), 6.95 (d, 2H, Ar-H, J=9.0), 7.31 (d, 4H, Ar-H, J=6.52), 7.45 (d, 2H, Ar-H, J=9.0); $^{13}$C NMR (400 MHz, CDCl$_3$) δ 21.2, 55.6, 114.8, 126.2, 127.1, 128.2, 130.1, 139.5, 150.9, 158.8; ESI-HRMS (M+H)$^+$ m/z calcd 314.36 for C$_{15}$H$_{12}$N$_2$O$_3$S; found 315.1.
3.2.3.4. **2,4-bis(4-methoxyphenyl)-1,2,4-thiadiazole-3,5-dione** (THIA IV)

Compound THIA IV was prepared from 4-methoxyphenyl-isothiocyanate (1g, 6.1mmol) and 4-methoxyphenyl-isocyanate (0.785mL, 6.1mmol) according to a procedure similar to that described above. Yield=71%; mp 177.8−177.3 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 3.83 (s, 3H, -OCH$_3$), 3.84 (s, 3H, -OCH$_3$), 6.95 (d, 2H, Ar-H, J=7.04), 7.01 (d, 2H, Ar-H, J=7.43), 7.34 (d, 2H, Ar-H, J=6.65), 7.45 (d, 2H, Ar-H, J=7.05); $^{13}$C NMR (400 MHz, CDCl$_3$) δ 55.5, 55.6, 114.7, 114.8, 125.3, 126.2, 128.2, 128.6, 151.0, 158.8, 160.0, 165.2; ESI-HRMS (M+H)$^+$ m/z calcd 330.36 for C$_{16}$H$_{14}$N$_2$O$_4$S; found 331.1.

3.2.3.5. **2-(4-chlorophenyl)-4-(4-methoxyphenyl)-1,2,4-thiadiazole-3,5-dione** (THIA V)

Compound THIA V was prepared from 4-chlorophenyl-isothiocyanate (1g, 5.9mmol) and 4-methoxyphenyl-isocyanate (0.764mL, 5.9mmol) according to a procedure similar to that described above. Yield=76%; mp 182.7−183.4 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 3.83 (s, 3H, OCH$_3$), 6.95 (d, 2H, Ar-H, J=8.61), 7.40 (d, 2H, Ar-H, J=8.61), 7.44 (d, 2H, Ar-H, J=7.04), 7.49 (d, 2H, Ar-H, J=8.61); $^{13}$C NMR (400 MHz, CDCl$_3$) δ 55.6, 114.8, 126.4, 127.8, 128.6, 129.6, 131.1, 135.2, 150.5, 159.0, 164.8; ESI-HRMS (M+H)$^+$ m/z calcd 334.78 for C$_{15}$H$_{11}$ClN$_2$O$_3$S; found 335.8.

3.2.3.6. **2-cyclohexyl-4-(4-methoxyphenyl)-1,2,4-thiadiazole-3,5-dione** (THIA VI)

Compound THIA VI was prepared from cyclohexyl-isothiocyanate (1g, 7.1mmol) and 4-methoxyphenyl-isocyanate (0.917mL, 7.1mmol) according to a procedure similar to that described above. Yield=77%; mp 98.8−99.7 °C; $^1$H
NMR (400 MHz, CDCl$_3$) δ 1.18-1.41 (m, 6H, cyclohexane: (CH$_2$)$_3$), 1.78-1.89 (q, 2H, cyclohexane: CH$_2$), 2.25-2.31 (q, 2H, cyclohexane: CH$_2$), 3.81 (s, 3H, OCH$_3$), 4.18-4.23 (qt, 1H, cyclohexane-CH), 6.92 (d, 2H, Ar-H, J=11.7), 7.37 (d, 2H, Ar-H, J=8.7); $^{13}$CNMR(400MHz,CDCl$_3$)δ 25.2, 26.1, 29.1, 55.0, 55.6, 118.9, 129.5, 132.4, 150.5, 159.0, 164.7; ESI-HRMS (M+H)$^+$ m/z calcd 306.38 for C$_{15}$H$_{18}$N$_2$O$_3$S; found 307.1.

3.2.3.7. 2-benzyl-4-(4-methoxyphenyl)-1,2,4-thiadiazole-3,5-dione (THIA VII) 

Compound THIA VII was prepared from benzyl-isothiocyanate (1g, 6.7 mmol) and 4-methoxyphenyl-isocyanate (0.868mL, 6.7 mmol) according to a procedure similar to that described above. Yield=75%; mp 113.7-114.5 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 3.81 (s, 3H, OCH$_3$), 4.90 (s, 2H, N-CH$_2$), 6.92 (d, 2H, OCH$_3$, J=8.60), 7.24-7.39 (m, 5H, Ar-H); $^{13}$C NMR (400 MHz, CDCl$_3$) δ 46.2, 55.6, 114.7, 126.2, 128.1, 128.4, 128.8, 129.1, 135.1, 151.4, 158.8, 165.4; ESI-HRMS (M+H)$^+$ m/z calcd 314.36 for C$_{16}$H$_{14}$N$_2$O$_3$S; found 315.0.

3.3. Pharmacology

3.3.1. Cysteine surrogates: propargylamide and $n$-butylamide derivatives

3.3.1.1. Isolated rat aortic ring assay

CD-1 mice were sacrificed and thoracic aorta was rapidly dissected and cleaned from fat and connective tissue. For endothelium-denuded rings, the endothelial layer was removed by gently rubbing the internal surface of the
vascular lumen. Rings of 1.5–2 mm length were cut and mounted in isolated organ
baths filled with gassed Krebs solution (95% O₂ + 5% CO₂) at 37 °C linked to
isometric force transducers (Ugo Basile, Comerio, Varese, Italy). The composition
of the Krebs solution was as follows (mol/l): NaCl 0.118, KCl 0.0047, MgSO₄
0.0023, KH₂PO₄ 0.0012, CaCl₂ 0.0025, NaHCO₃ 0.025 and Glucose 0.010. Rings
were initially stretched until a resting tension of 1.5 g was reached and allowed to
equilibrare for at least 45 min during which tension was adjusted, when necessary,
to 1.5 g and bathing solution was periodically changed. In a preliminary study a
resting tension of 1.5 g was found to develop the optimal tension to stimulation
with contracting agents.

In each experiment, rings were firstly contracted with L-phenylephrine
(PE) 3 μmol/l and subsequently with L-phenylephrine (PE) 1 μmol/l until the
response were reproducible. To verify the integrity of the endothelium, Ach
cumulative concentration-response curve (10nM – 30μM) was performed on PE-
contracted rings.

Aortic rings were contracted with PE (1μM), once plateau was reached, a
cumulative concentration-response curve to surrogate cysteine or derived
compounds (10nM – 300μM) was performed.

In another set of experiments aortic rings were incubated with the cysteine
surrogates or with their derivatives at different concentration (1mM or 100μM)
for 30 minutes. After that time, rings were contracted with PE (1μM), once
reached a plateau, a cumulative concentration-response curve to L-cysteine (1μM-
3 mM) was performed.
All data are presented as means ± SEM. Statistical analysis was performed by one-way ANOVA followed by Newman–Keuls multiple comparison test. Differences were considered statistically significant when P-value was less than 0.05. GraphPadPrism software (GraphPad Software, Inc., La Jolla, CA, USA) was used for all the statistical analysis.

3.3.1.2. Measurement of H₂S production: methylene blue assay

H₂S determination was performed according to Stipanuk and Beck (Stipanuk and Beck, 1982) with modifications.

Briefly, thoracic aorta was dissected, placed in sterile phosphate buffer solution and cleaned of fat and connective tissue. The aortic rings were homogenized in a lysis buffer (potassium phosphate buffer 100 mM pH=7.4, sodium orthovanadate 10 mM and protease inhibitor) and the protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Milano, Italy). The homogenates were treated with cysteine surrogates and their derived compounds at different concentration for 30 minutes and then added in a reaction mixture (total volume 500µl) containing piridoxal-5’-phosphate (2mM, 20µl), L-cysteine (10mM, 20µl) and saline (30µl). The reaction was performed in parafilmed eppendorf tubes and initiated by transferring tubes from ice to a water bath at 37°C. After incubation of 30 minutes, ZnAc (1%, 250µl) was added to trap evolved H₂S followed by TCA (10%, 250µl). Subsequently, N,N-dimethyl-p-phenylenediamine-sulfate (20µM, 133µl) in 7.2M HCl and FeCl₃ (30µM, 133µl) in 1.2M HCl were added. After 20 minutes absorbance values were measured at a wavelength of 650 nm. All samples were assayed in duplicate and H₂S
concentration was calculated against a calibration curve of NaHS (3.12-250µM). Results were expressed as nmoles per milligram of protein.

3.3.2. Glucocorticoids-\(\text{H}_2\text{S}\) donors

3.3.2.1. Cell cultures and Nitrite determination

The murine monocyte/macrophage J774 cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM glutamine, 25 mM hepes, penicillin (100 units/mL), streptomycin (100 µg/mL), 10% fetal bovine serum (FBS), and 1.2% sodium pyruvate.

Cells were plated to a seeding density of either 2 x 10^6 in P60 well plates or 2.5 x 10^5 in 24 multiwell plates. Cells were pretreated (for 2h) with increasing concentration of the tested compounds and stimulated with LPS from \(\text{Escherichia coli}\), serotype 0111:B4 (10 µg/mL). Treatment with tested compounds and/or LPS was carried out under serum-free conditions. The concentrations of the FAS utilized (0.1 and 1 µg/mL) were chosen according to what was reported in the literature.

Cells were pretreated with tested compounds for 2h and further incubated for 24 h with LPS (10 µg/mL). At the end of the incubation, the supernatants were collected for the nitrite measurement.

The nitrite concentration in the samples was measured by the Griess reaction, by adding 100 µL of Griess reagent (0.1% N-(1-naphthyl)ethylenediamide dihydrochloride in \(\text{H}_2\text{O}\) and 1% sulfanilamide in 5% \(\text{H}_3\text{PO}_4\); vol. 1:1) to 100 µL samples. The optical density at 550nm (OD_{550}) was measured using an ELISA microplate reader (SLT Lab instruments, Salzburg,
Austria). Nitrite concentration was calculated by comparison with OD550 of standard solutions of sodium nitrite prepared in culture medium.

3.3.3. 1,2,4-Thiadiazolidine-3,5-diones

3.3.3.1. Amperometric determination of H2S

The characterization of the potential H2S-generating properties of the tested compounds has been carried out by an amperometric approach, using the Apollo-4000 free radical analyzer (WPI) detector and H2S-selective mini-electrodes.

The experiments have been carried out at room temperature. The H2S-selective mini-electrode was equilibrated in 10 mL of the assay buffer (NaH2PO4·H2O 1.28 g, Na2HPO4·12H2O 5.97 g, NaCl 43.88 g in 500 mL H2O), until the recovery of a stable baseline. Then, 100 μL of a DMSO solution of all the tested THIAs was added (the final concentration of the tested compounds was 1 mM; the final concentration of DMSO in the AB was 1%).

The selected compounds were tested in absence and in presence of L-cysteine and when required by the experimental protocol, L-cysteine (4 mM) was added before the addition of the THIAs. The H2S generation was monitored for 20 min. H2S concentration was determined against a calibration curve obtained plotting amperometric currents (recorded in pA) vs. increasing concentrations of NaHS (1, 3, 5, and 10 μmol/L) at pH 4.0.

3.3.3.2. Isolated rat aortic ring assay

CD-1 mice were sacrificed and thoracic aorta was rapidly dissected and cleaned from fat and connective tissue. For endothelium-denuded rings, the endothelial layer was removed by gently rubbing the internal surface of the
vascular lumen. Rings of 1.5–2 mm length were cut and mounted in isolated organ
baths filled with gassed Krebs solution (95% O₂ + 5% CO₂) at 37 °C linked to
isometric force transducers (Ugo Basile, Comerio, Varese, Italy). The composition
of the Krebs solution was as follows (mol/l): NaCl 0.118, KCl 0.0047, MgSO₄
0.0023, KH₂PO₄ 0.0012, CaCl₂ 0.0025, NaHCO₃ 0.025 and Glucose 0.010. Rings
were initially stretched until a resting tension of 1.5 g was reached and allowed to
equilibrane for at least 45 min during which tension was adjusted, when necessary,
to 1.5 g and bathing solution was periodically changed. In a preliminary study a
resting tension of 1.5 g was found to develop the optimal tension to stimulation
with contracting agents.

In each experiment, rings were firstly contracts with L-phenylephrine (PE) 3
µmol/l and subsequently with L-phenylephrine (PE) 1 µmol/l until the response
were reproducible. To verify the integrity of the endothelium, Ach cumulative
concentration-response curve (10nM – 30µM) was performed on PE-contracted
rings.

Aortic rings were contracted with PE (1µM), once plateau was reached, a
cumulative concentration-response curve to THIA (10nM – 300µM) was
performed. In another set of experiments, rings were denuded of the endothelium
and THIA cumulative concentration-response curve was performed (10nM –
300µM).
4. Results and Discussion

4.1. Cysteine surrogates: propargylamide and n-butylamide derivatives

In order to evaluate the inhibitory activity of the selected compounds, we firstly tested the commercially available cysteine surrogates on the L-cysteine-induced vasorelaxation in rat aortic rings and compared the results to the effect obtained with synthesized compounds. In physiological conditions, L-cysteine induced vasorelaxation, in a concentration-dependent manner, with a maximum value of 39.8 ± 5%.

The pre-treatment with the compound II reduced the L-cysteine-induced vasorelaxation in a concentration-dependent manner. Indeed, the tested compound at the concentration of 1 mM reduced the vasorelaxation significantly and at the concentration of 3 mM achieved the E\textsubscript{max} value of 13.7 ± 6.1% (n=3;***=p<0.001). In contrast, the treatment with the compound II (Figure 11) tested at the concentration of 100 µM did not affect the L-cysteine-induced vasorelaxation (E\textsubscript{max} 27.7±11.1% II vs 39.8±5% vehicle respectively; n=3).

Figure 12 shows the results obtained with the compound III. In particular, this compound, tested at the concentration of 10 mM, significantly reduced the L-cysteine-induced vasorelaxation, (E\textsubscript{max} 26±5% III vs 39.8±5% vehicle; n=4). This effect was concentration-dependent, in fact, the pre-treatment with the compound III at the concentration of 1 µM, did not affect the L-cysteine-induced vasorelaxation (E\textsubscript{max} 26 ± 5 % III vs 39.8 ± 5 % vehicle; n = 4).
In contrast, as shown in figure 10, the pre-treatment with the compound I induced a significant increase of the L-cysteine induced vasorelaxation effect (E_{max} 74.0 \pm 17.6\% \text{ I vs 39.8 \pm 5\% vehicle}; \ n = 4; \ *** = p < 0.001).

Incubation of aortic rings with the compound IIa at the concentration of 100 \mu M (Figure 14) completely abrogated the L-cysteine-induced vasorelaxation, achieving the E_{max} value of 13.7 \pm 6.1\%(n=3;***=p<0.001), but unaffected by Ia (Figure 13).

A similar pattern of inhibition was obtained with the compound IIIa (Figure 15). In particular L-cysteine-induced vasorelaxation was reduced by pre-treatment with the compound IIIa. However, compared to IIa, the compound IIIa, even at the highest concentration tested (1mM), was not able to completely abrogate the relaxation (E_{max} 13.3\pm1.8\% \text{ IIIa 1mM; 24.0\pm2.0 IIIa 100 \mu M, vs 39.8\pm5\% vs vehicle; n=3; * p< 0.05; ** p< 0.01}).

Furthermore, the synthesized compounds were also tested for their potential as vasorelaxing agents on rat aortic rings and these relaxations were compared to those obtained in the presence of L-cysteine (Figure 16). Compounds Ia and IIa have also showed a vasorelaxing ability that was similar or slightly higher than the L-cysteine (E_{max} 51.3\pm14.8\% \text{ IIa; 49\pm10.0\%IIa; 31\pm16.1\% vs 38.8\pm9.5\%Ia vs L-cys; n=3}).

In another set of experiments, n-butylamide derivatives (Ib-IIIb) were tested for their ability to inhibit the L-Cys induced vasorelaxation on rat aortic rings pre-contracted with PE but none of tested compounds affected the vasorelaxation effect (data not shown).
With regard to parasalmide (IV) and its derivatives (IVa and IVb), all of them significantly inhibited L-cysteine induced vasorelaxation (Figure 17) and IV and IVb also showed vasorelaxant activities (Figure 18).

Additionally, in order to evaluate a possible involvement of L-Cys/H₂S pathway, the compounds showing the higher inhibitory action, were tested by measuring H₂S production and the methylene blue assay was performed.

Under basal conditions, tissue homogenates produced 1,4 ± 0,08 % nmol of H₂S per mg of protein and the addition of exogenous L-Cys boosted the production of H₂S by 2-fold.

Incubation with compounds II, III, IIa and IIIa significantly inhibited the increase in H₂S production stimulated with L-Cys. These results confirmed those obtained from ex vivo experiments. In fact, as shown in figure 20, pre-treatment with the propargylamide derivative compounds (IIa, 100 μM and IIIa, 1mM) reduced H₂S production compared to the vehicle (Eₘₐₓ 2,7±0,09 nmol/mg).

Comparing the compounds IIa (Eₘₐₓ 2,1±0,07% nmol/mg protein⁻¹(min⁻¹)(n=4)) and IIIa (Eₘₐₓ 1,960±0,06% nmol/mg protein⁻¹(min⁻¹)(n=3)), they showed a comparable inhibitory action on H₂S production, but the effect of the compound IIa was obtained with a lower dose (Ia, 100 μM vs IIIa, 1mM).

On the basis of the obtained results, we decided to compare the inhibitory activity of the compound IIa with the known CSE inhibitor, D,L-propargylglycine (PGG) (Figure 19).

Incubation of aortic rings with the compound IIa completely abrogated the L-cysteine-induced vasorelaxation, achieving the Eₘₐₓ value of 3,7±0.88%(n=3;***=p<0,001), while PGG reduced but not completely abrogated
the L-cysteine-induced vasorelaxation ($E_{\text{max}} = 20.3\pm5.2\%\text{nmol/mg protein min}^{-1}$ (n=3) vs $45.3\pm6.7\%\text{nmol/mg protein min}^{-1}$ vehicle), despite it was tested at an higher concentration (1mM vs 100µM; PGG vs IIa).

**Figure 10**- Effect of compound I on L-cysteine-induced vasorelaxation.

**Figure 11**- Effect of compound II on L-cysteine-induced vasorelaxation.
Figure 12- Effect of compound III on L-cysteine-induced vasorelaxation.

Figure 13- Effect of compound Ia on L-cysteine-induced vasorelaxation.
**Figure 14** - Effect of compound IIa on L-cysteine-induced vasorelaxation.

**Figure 15** - Effect of compound IIIa on L-cysteine-induced vasorelaxation.
Figure 16- Effect of propargylamide derivatives (Ia-IIIa), compared to L-cysteine, on vasorelaxation.

Figure 17- Effect of parsalmide (IV) and derivatives (IVa-IVb) on L-cysteine-induced vasorelaxation.
Figure 18- Effect of propargylamide derivatives (Ia-IIIa), compared to L-cysteine on vasorelaxation.

Figure 19- Effect of compound IIa, compared to the CSE inhibitor PGG on L-cysteine-induced vasorelaxation.
**Figure 20**- Measurement of H$_2$S production in rat aortic rings homogenates with II, III, IIa and IIIa compounds, compared to PGG.

### 4.2. Glucocorticoids-H$_2$S donors

Asthma is one of the most common chronic inflammatory disorder of the airways of the lungs. Nitric oxide (NO) is endogenously produced in mammalian airways by nitric oxide synthase (NOS) and is known to regulate many aspects of human asthma, including the modulation of airway and vascular smooth muscle tone and the inflammation. Asthmatic patients show an increased expression of inducible nitric oxide synthase (iNOS) in airway epithelial cells and an increased level of NO in exhaled air. iNOS produces large amounts of NO (Alderton et al., 2001), in response to inflammatory signals, such as cytokines and lipopolysaccharides (LPS).

The glucocorticoids have been reported to inhibit iNOS induction, which may contribute to the inflammation-reducing effects. Specifically, glucocorticoids
(10-250 nM) inhibited the expression of iNOS mRNA and protein dose and time
dependently, resulting in decreases in NO production.

On the other hand, a growing number of observations suggest that, similarly
to NO, hydrogen sulfide might be of biological relevance as an endogenous
gasotransmitter in the pathogenesis of airway diseases, such as COPD and asthma
(Chen and Wang, 2012). Endogenous H$_2$S may exert its anti-inflammatory effect
by inhibiting inducible nitric oxide synthase (iNOS)/NO pathway. Cystathionine-
$\gamma$-lyase is mainly expressed in airway and vascular smooth muscle cells in rat lung
tissue.

For these reasons, we tested the synthetized hybrids on J774 macrophages
stimulated with LPS (10 $\mu$g/mL), measuring the NO production and comparing
obtained data to the pure glucocorticoids (dexamethasone and beclomethasone
succinates).

In J774 cells stimulated with LPS, pure glucocorticoids clearly inhibited NO
formation in a concentration-dependent manner, as shown in Figure 21 and 22,
comparing two sets of data at different concentration (log M $-7$ vs. log M $-6$).

When succinate glucocorticoids and derivatives were given to cells 2 h
before LPS, the inhibitory activity of dexamethasone succinate was lower than its
derivatives, glucocorticoid-H$_2$S donors (FAS I and II), but this effect did not
occur with beclomethasone derivatives. Specifically, pretreatment with FAS III
and IV 2 h before LPS did not induce any inhibition (Figure 21).

These data suggested that a pre-incubation of 2 h was not enough to lead to
the hydrolysis of ester bond in beclomethasone-hybrids, allowing the release of
the pure glucocorticoid and its H$_2$S donor moieties.
Consequently, another set of experiments was performed and J774 cells were pretreated for a longer time (4h). The obtained data confirmed the enhanced inhibitory activities of dexamethasone-H$_2$S donors (FAS I and II), compared to the precursor, dexamethasone succinate (Figure 22).

These results supported our hypothesis that the anti-inflammatory effect could be enhanced with hybrids containing glucocorticoids and H$_2$S releasing moiety, both responsible for inhibitory action on inducible nitric oxide synthase.

In contrast, the effect of beclomethasone succinate on nitrite production, even at the highest concentration used (1µM), resulted equal or greater than that obtained with its derivatives (FAS IV and III, respectively).

This result can be explained by the presence of a chlorine atom into the 9-position of a pregnane steroid, instead of fluorine, that could have a long range effect on ester bond, slowing down the hydrolysis and the subsequent hydrogen sulfide release.

![Figure 21](image)

**Figure 21** - Effect of glucocorticoids and derivatives on nitrite production. J774 macrophages were pretreated for 2 h with glucocorticoids and derivatives.
Figure 22 - Effect of glucocorticoids and derivatives on nitrite production. J774 macrophages were pretreated for 4 h with glucocorticoids and derivatives.

4.3. 1,2,4-Thiadiazolidine-3,5-diones

In order to characterize the potential H\textsubscript{2}S-releasing properties of 1,2,4-thiadiazolidine-3,5-diones, all compounds were added at 1 mM concentration to the assay buffer. The generation of H\textsubscript{2}S was evaluated by an amperometric approach, allowing to have a real-time determination of the H\textsubscript{2}S-release and thus to perform a qualitative/quantitative description of the process.

The experiments were also performed in the presence of 4 mM L-cysteine. The incubation of THIA I-VII in the assay buffer led to a negligible release of H\textsubscript{2}S (data not shown); in contrast, in the presence of L-cysteine, all compounds (VII except) exhibited a slow and significant release of H\textsubscript{2}S (Figure 23).

Noteworthy, the previous amperometric measurements indicated that THIAs ensured a long-lasting release of H\textsubscript{2}S, requiring the presence of L-cysteine.
Figure 23 - Amperometric determination of H$_2$S. Hydrogen sulfide-release from THIAs in the presence of L-cysteine. The curves describe the increase of the H$_2$S concentration with respect to time, following the incubation of THIA I-VII in the presence of L-cysteine.

Additionally, to prove the nature of 1,2,4 thiadiazolidine 3,5 diones as spontaneously releasing H$_2$S donors, further pharmacological experiments were performed. In these studies, the endothelium-intact or endothelium-denuded aortic rings were exposed to the THIA I-VII.

The tested compounds induced a vasorelaxant effect, irrespective of whether endothelium was present or not. In particular, incubation of aortic rings with THIA I (Figure 24), II (Figure 25), III (Figure 26), V (Figure 28) and VI (Figure 29) allowed us to obtain the maximum vasorelaxant effect, that was independent of endothelium.

Furthermore, the pre-treatment of endothelium-intact aortic rings with THIA IV induced a maximum vasorelaxation, as well as the other compounds of the series, but the response was reduced in endothelium-denuded rings (75%)
(Figure 27). Instead, the removal of the endothelium showed an increase of the vasorelaxing capacity of THIA VII (Figure 30). The obtained results suggested that the presence of endothelium with all their components could affect the release of the hydrogen sulfide.

Figure 24 – Effect of THIA I compound on endothelium-denuded and endothelium-intact aortic rings.
Figure 25 – Effect of THIA II compound on endothelium-denuded and endothelium-intact aortic rings.

Figure 26 – Effect of THIA III compound on endothelium-denuded and endothelium-intact aortic rings.
Figure 27 – Effect of THIA IV compound on endothelium-denuded and endothelium-intact aortic rings.

Figure 28 – Effect of THIA V compound on endothelium-denuded and endothelium-intact aortic rings.
Figure 29 – Effect of THIA VI compound on endothelium-denuded and endothelium-intact aortic rings.

Figure 30 – Effect of THIA VII compound on endothelium-denuded and endothelium-intact aortic rings.
5. NMR-based metabolomics

5.1. Objectives

In order to confirm the promising results obtained in *ex vivo* tissues with the synthesized compound IIa, able to completely inhibit L-cysteine-induced vasorelaxation, the effects of the selected compound were further tested on the purified recombinant enzymes.

For this purpose, a metabolomic approach based on nuclear magnetic resonance techniques was used, upon expression, purification and characterization of CBS and CSE enzymes.

5.2. Expression and purification of recombinant CBS and CSE enzymes.

5.2.1. Plasmids, bacterial strains and media

E. coli BL21 (DE3) Codon Plus was used as the host strain to express recombinant human CSE or CBS. CSE cDNA was cloned into pGEX-4T3 and CBS into pGEX-Kg to create N-terminal GSH-S-transferase (GST) fusion proteins. The expression vectors were transformed and plated on LB-agar plates, supplemented with ampicillin (100 mg·mL⁻¹).

5.2.2. Bacterial expression of human CBS enzyme

The expression and purification of CBS was performed as described previously with modifications (Huang, 2010).

A colony of the pGEX-Kg/GST-CBS transformed BL21 cells was
inoculated into 100 mL of steam-autoclaved Luria-Bertani (LB) broth supplemented with 100 μg/mL of ampicillin (LB-Amp100), and incubated at 37 °C overnight with vigorous shaking.

A part of starter culture (40 mL) was propagated into 5 L baffled flask, containing 960 mL of LB-Amp100. The mixture was incubated at 37 °C with vigorous shaking until an optical density of 0.3 at 600 nm (OD$_{600}$) was attained, upon which bacterial growth was continued at 30 °C until an OD$_{600}$ of 0.5 was attained.

After the addition of 0.3 mM δ-ALA, IPTG was added at a rate of 0.1 mM to induce the expression of human CBS at 30 °C for 18 h. The cells were harvested by centrifugation in a Beckman Avanti centrifuge J-26XP equipped with a JSP-FIBERLite rotor at 5000 rpm for 40 min at 4 °C. The supernatants were thrown away and the pellets, re-suspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.3), were kept at -20 °C overnight.

5.2.3. Bacterial expression of human CSE enzyme

The expression and purification of CSE was adapted from a previously described (Huang, 2010).

A colony of the pGEX-4T-3/GST-CSE transformed BL21 cells was inoculated into 100 mL of steam-autoclaved Luria-Bertani (LB) broth supplemented with 100 μg/mL of ampicillin (LB-Amp100), and incubated at 37 °C overnight with vigorous shaking.

A part of starter culture (40 mL) was propagated into 5 L baffled flask, containing 960 mL of LB- Amp100. The mixture was incubated at 37 °C with
vigorously shaking until an optical density of 0.3 at 600 nm (OD\textsubscript{600}) was attained,

upon which bacterial growth was continued at 18 \textdegree C until an OD\textsubscript{600} of 0.5 was attained. IPTG was then added at a rate of 0.1 mM to induce the expression of human CSE at 18 \textdegree C for 18 h. The cells were harvested by centrifugation in a Beckman Avanti centrifuge J-26XP equipped with a JSP-FIBERLite rotor at 5000 rpm for 40 min at 4 \textdegree C.

The supernatants were thrown away and the pellets, re-suspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.8 mM KH\textsubscript{2}PO\textsubscript{4}, pH 7.3), were kept at -20 \textdegree C overnight.

**5.2.4. Purification of human enzymes**

The bacterial pellets were thawed and re-suspended in lysis buffer containing PBS pH 7.3, 1\% Igepal, 5 mM dithiothreitol (DTT), 100 mM PLP, protease inhibitors (Complete EDTA-free tablet), DNAse I and Lysozyme. Cell lysis was performed by sonication on ice using the 30\% pulsed maximum output of a Brandson Sonifier 250 equipped with a macrotip. The lysates were then cleared by centrifuging at 16000 rpm for 30 min at 4 \textdegree C using a Beckman JA20 rotor.

The soluble fractions, containing the GST-CBS and GST-CSE recombinant proteins respectively, were loaded onto a Glutathione sepharose 4B columns previously equilibrated with binding buffer PBS. The columns were sealed and incubated with slight shaking in a cold room (4 \textdegree C) for 20 min.

Following that, the flow-throughs were collected, and the beads were washed with five column volumes of binding buffer. Proteins attached to the
columns were eluted with five column volumes of elution buffer (50 mM Tris–HCl, 10 mM reduced GSH, 5 mM DTT, pH 8.0) and then dialyzed and concentrated in 10 mM sodium phosphate buffer pH 8.2 and DTT 1 mM.

This was repeated until minimal protein concentration was detected in the eluates by Bradford assay. The eluates were then pooled together and concentrated using an ultra-centrifugal filter (Millipore Amicon Ultra-4 30000 MWCO) at 4 °C.

The concentrated eluates were subsequently passed through a Superdex-200 column connected to the AKTA purifier FPLC equilibrated with buffer A (10 mM sodium phosphate buffer pH 8.2 and 1 mM DTT) at a flow rate of 1 mL/min (Figure 31).
B.

**Figure 31 - FPLC chromatography.** Gel filtration profile of GST-CBS (A) and GST-CSE (B) proteins.

Fractions containing CBS and CSE respectively were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then pooled together. Peak fractions corresponding to the target CBS and CSE proteins were pooled together and concentrated to about 5 mg/mL in 10 mM sodium phosphate buffer pH 8.2 and 1 mM DTT using an ultra-centrifugal filter (Millipore Amicon Ultra-4 10000 MWCO) at 4 °C. The proteins were kept at -80 °C for long-term storage.

The purity of the recombinant enzymes was checked by SDS-PAGE on 12% polyacrylamide gels after staining of protein bands with Coomassie dye (**Figure 32**).

GST was not removed from the fusion proteins as it has been previously
reported that the presence of GST does not affect activity. Furthermore, GST did not interfere with the assay, since no H₂S-synthesizing activity was observed in a control activity experiment with GST alone (Huang, 2010).

**Figure 32 - Representative SDS-PAGE.** Fractions after different purification steps of recombinant GST-CBS (a) and GST-CSE (b) from bacterial cell lysates. Lanes: M, protein marker; 1, cell Lysate before purification; 2. flow through; 3. wash with binding buffer; 4. eluted GST-CBS or GST-CSE; 5. purified proteins after gel filtration.
5.3. Structural characterization of recombinant enzymes

5.3.1. Analysis of protein secondary structure via circular dichroism (CD) measurements

Far-UV CD measurements were performed on a J-715 spectropolarimeter (JASCO, Oklahoma City, OK) equipped with a PTC-348 Peltier temperature control system. The instrument was flushed with nitrogen gas, after which the nitrogen gas flow was maintained at a flow rate of 4 L/min. Samples were prepared using an enzyme concentration of 4 µM in 10 mM sodium phosphate pH 8.2 buffer. The spectra were recorded at different time points using quartz cuvettes (Hellma, Plainview, NY) with path-lengths of 1 mm.

Baseline correction was performed by subtraction of the appropriate buffer spectrum. The recorded CD spectra resulted from averaging five separate wavelength scans on independent protein preparations to ensure reproducibility of the results. The parameters were scanned wavelength from 260 nm to 190 nm, scanning speed 200 nm/min, data pitch 0.2 nm, 2 nm slit width, temperature 37 °C for each protein (Figure 33).

CD intensities are presented as the CD absorption coefficient calculated using the molar concentration of the proteins ($\Delta\varepsilon_M$). Far-UV CD spectral decomposition to obtain the secondary structure content was achieved with the CONTIN, SELCON, and CDSSTR methods (Figure 34).
Figure 33 - Circular dichroism. Far-UV CD spectra of GST-CBS (A) and GST-CSE (B) in 10 mM sodium phosphate pH 8.2
Figure 34 - Circular dichroism, the secondary structure content. Proportion of α-helices, β-sheets, turns and unordered regions of GST-CBS (A) and GST-CSE (B) achieved with the CONTIN, SELCON, and CDSSTR methods.
5.3.2. UV-vis Spectroscopy Analysis of recombinant enzymes CBS and CSE

Adsorbance spectroscopy was carried out on a Varian Cary 50 Bio UV-Visible Spectrophotometer. The analyzed samples were dissolved in 10 mM sodium phosphate pH 8.2.

Measurements were performed in a 1 cm cuvette cell and monitored spectroscopically from 650nm to 250 nm. The absorption spectrum of human CBS exhibited a peak at 430 nm (Figure 35A) suggesting the presence of haem in the recombinant protein. As shown in Figure 35B, the purified CSE enzyme displayed the characteristic 427 nm absorbance peak which corresponded to the Schiff-base linkage between PLP and Lys-212.

A.
5.3.3. Kinetics of $\text{H}_2\text{S}$ production from purified recombinant human CBS and CSE

5.3.3.1. Determination of $\text{H}_2\text{S}$ standard curve

A mixture of 0.85 % w/v ZnAc and 3 % w/v NaOH (100 $\mu$L) was added to various $\text{H}_2\text{S}$ standards (0-60 $\mu$M, 100 $\mu$L) prepared in 50 mM sodium phosphate pH 8.2 buffer containing 0.5 mM PLP, 20 mM NaCl and 0.1 mM DTT. TCA (10 % w/v, 100 $\mu$L) was added and the tubes were then treated with NNDPD (20 mM, 42.9 $\mu$L) and FeCl$_3$ (30
mM, 42.9 μL). After a short spin, 300 μL of the supernatant was removed for absorbance measurements at 670 nm.

The calibration curve was generated by plotting the average $A_{670}$ value against the amount of $H_2S$ present for each standard (Figure 36).

A.
Figure 36 - H$_2$S standard curve. A. UV-vis spectra of H$_2$S standards using the methylene blue assay. B. Calibration curve of the spectrophotometric detection of hydrogen sulfide.

5.3.3.2. Enzyme activities

In order to verify the catalytic activities of the purified enzymes, hydrogen sulfide production was measured by the methylene blue method. The assay was performed according to Stipanuk and Beck (1982) with some modifications.

Each reaction mixture consisted of 5 μg of the purified enzyme, saline (10 μL), and L-cysteine (27.5 mM, 10 μL), dissolved in 10mM sodium phosphate pH 8.2 buffer to a final volume of 100 μL. For the CBS enzyme, the reaction mixture contained the same as for the CSE plus 27.5 mM homocysteine (10 μL).

The Eppendorf tubes were parafilmed tightly, gently vortexed, and then incubated in a 37 °C water bath. After 60 min of incubation, the evolved H$_2$S was
trapped via addition of ZnAc (1 % w/v, 100 μL) and proteins were precipitated via addition of TCA (10 % w/v, 100 μL).

![Reaction of methylene blue assay](image)

**Figure 37 - Reaction of methylene blue assay**

Subsequently, N,N-dimethyl-p-phenylenediamine-sulfate in 7.2 M HCl was immediately followed by addition of FeCl₃ in 1.2 M HCl, for development of methylene blue, according to the reaction equation shown above (Figure 37).

The amount of H₂S produced was then determined via absorbance measurements of the centrifugal supernatants. The absorbance of the resulting solution was measured at 655 nm (Figure 38).
Figure 38 - Spectrophotometric method using methylene blue assay. Measurement of H$_2$S producing CBS (A) and CSE enzymes (B).
5.4. Enzyme Kinetics: NMR spectroscopy

Metabolomics Nuclear Magnetic Resonance technique was used to study the kinetics of two main enzymes involved in trans-sulfuration pathway. Specifically, CBS converts L-cysteine and L-Homocysteine into L-cystathionine (Figure 39A) and CSE enzyme utilizes L-cysteine as a substrate in an α,β-elimination reaction to produce H₂S, pyruvate and ammonia (Figure 39B). NMR spectra were acquired to follow the disappearance of the L-Cysteine peaks over time, monitoring its conversion in cystathionine or pyruvate, the final products of CBS and CSE enzymes, respectively.

![Figure 39A](image1.png)

**Figure 39- Enzyme kinetics.** Reactions catalyzed by cystathionine beta synthase (A) and cystathionine gamma lyase (B).

5.4.1. Sample preparation

All reaction components were prepared in 10 mM sodium phosphate pH 8.2. A standard assay in a 5 mm glass tube was composed of the enzyme substrate, \(^{15}\text{N,}^{13}\text{C}-\text{L-Cysteine} (125 \mu\text{L}, 1 \text{mM}), \text{L-Homocysteine} (125 \mu\text{L}, 1 \text{mM})\) (added
only for CBS timecourse as co-substrate), DTT (0.5 µL, 1M), D₂O (50 µL), enzyme (100 µL, 110 µM) and filled to a final volume of 500 µL with 10 mM sodium phosphate buffer pH 8.2 in a 5 mm NMR tube. In order to verify the enzyme kinetics in the presence of the potential inhibitor, all components above were put into a different tube, preincubating the selected compound IIa (125 µL, 2.5 mM) with each enzyme. Similarly, another sample was prepared using the known CSE inhibitor, D.L-propargylglycine (PGG) (125 µL, 2.5 mM), instead of the compound IIa.

5.4.2. NMR measurements

NMR experiments were performed at 300 K on a Bruker AVANCE spectrometer operating at 800 MHz with TCI Cryoprobes. Initially, the isolated enzyme or the enzyme pre-incubated with the tested compounds was excluded from the sample as a control. This blank was used to tune the spectrometer, acquire a lock signal and shim the instrument before the reaction was started by removing the tube from the instrument, adding the enzyme (100 µL, 110 µM), mixing several times by inverting the tube and re-inserting the tube in the instrument. Data acquisition was initiated once a stable lock signal was achieved.

The same set of experiments was carried out in the absence and in the presence of tested compound (compound IIa and PGG, separately), pre-incubated with each enzyme.

For each sample, pseudo-2D experiments were collected in which the first increment of a ¹³C HSQC spectrum was acquired with a 5 minutes repetition time for a total of 60 spectra, using ¹⁵N, ¹³C, L-Cysteine and unlabeled L-Homocysteine.
as CBS substrate and $^{15}$N,$^{13}$C, L-Cysteine as a unique substrate for CSE enzyme. Furthermore, 2D $^1$H,$^{13}$C HSQC spectra were acquired before and after the enzyme reaction was completed.

5.5. Results and Discussion II

Pseudo 2D spectra allowed us to follow $^{15}$N,$^{13}$C, L-Cysteine conversion into the final products. Two dimensional $^{13}$C HSQC spectra were collected before and after each experiment to further confirm the completeness of the reactions.

The time course for CBS production of L-Cystathionine is presented in Figure 40. As shown, $^{15}$N,$^{13}$C, L-Cysteine peaks ($^1$H-NMR $\delta$ 3.05, C$_{\beta}$H$_2$; 3.90, C$_{\alpha}$H) were gradually decreasing until completely disappearance, while the product was growing up. Cystathionine peaks ($^1$H-NMR $\delta$ 3.97, 3.15, 3.10) were not all detected, because of the unlabeled homocysteine contribution.

With regard to the time course of CSE, Figure 41 clearly shows that $^{15}$N,$^{13}$C, L-Cysteine peaks ($^1$H-NMR $\delta$ 3.05, C$_{\beta}$H$_2$; 3.90, C$_{\alpha}$H) decrease in size until completely disappearance, whereas the product pyruvate increase ($^1$H-NMR $\delta$ 2.4, CH$_3$).

Another set of experiments was carried out in the presence of compound II, pre-incubated with each enzyme. When compound IIa was incubated with CBS enzyme, there was no kinetic modification, with the complete conversion of L-Cysteine into L-cystathionine (Figure 42). Figure 48 shows 3D graphics derived from all acquired 1D $^{13}$C HSQC spectra over time during CBS time course (A) and the kinetics in the presence of compound IIa (B); comparison of the resulting graphics showed no significant differences.
Conversely, the tested compound was able to inhibit the production of pyruvate catalyzed by CSE (Figure 43), proving to be a CSE selective inhibitor, as well as the known CSE inhibitor, D,L-propargylglycine (PGG). It is clearly evident in the Figure 49 representing 3D graphics derived from all acquired 1D $^{13}$C HSQC spectra over time during CSE timecourse (A) and the kinetics in the presence of compound IIa (B).

To verify if it is a competitive reaction, at the end of the CSE time course with compound IIa, an excess of L-cysteine was added and the experiment was carried out again. As shown in Figure 50, the cysteine peaks were not decreased, demonstrating that the enzyme was not able to continue its work anymore and that it was a noncompetitive inhibition.

When PGG was used in the enzyme reaction, the pyruvate peak did not increase, as expected. Surprisingly, the L-cysteine peaks decreased and new product peaks, at 3.92, 3.40, 3.25 ppm, different from pyruvate, appeared and progressively increased (Figure 44).

Most likely the new peaks represent the cystine. A possible explanation is that the cystine was formed enzymatically and not chemically, because in the reducing environment for the presence of dithiothreitol (DTT), it would not be possible the cysteine oxidation and consequent formation of the disulfide bond, unless enzyme activity.

To further verify the completeness of the reaction, two dimensional $^{13}$C HSQC spectra, acquired before and after each time course, were overlapped. Figure 45 shows the cysteine peaks (blue spectrum acquired before the reaction), appeared at 55 ppm in F1 and 3.90 ppm in F2 ($C_\alpha$H) and at 25 ppm in F1 and 3.05
ppm in F2 ($C_\beta H_2$). These peaks are absent in the $^{13}$C HSQC spectrum (red), acquired at the end of the reaction, confirming the completeness of reaction. In Figure 46 $^{13}$C HSQC spectrum acquired after CSE time course with compound IIa, was shown. The persistence of cysteine peaks at the end of the reaction confirms that compound IIa is a selective CSE inhibitor, by blocking the formation of pyruvate with a different mechanism from PGG inhibition. When CSE timecourse with PGG is completed, $^{13}$C HSQC spectrum (shown in Figure 47) was acquired. Pyruvate production was blocked (despite a very small amount was formed, as evidenced by the presence of pyruvate peak); however, cysteine peaks were decreased, converting mainly into cystine (peaks at 55 in F1 and 3.92 ppm in F2; 32 ppm in F1 and 3.4 in F2; 32 ppm in F1 and 3.25 ppm in F2). This suggests that both compounds (IIa and PGG) inhibit CSE production of pyruvate, by acting through two different mechanisms of inhibition: the compound IIa prevents the CSE enzyme from binding to its substrate, inhibiting the conversion of L-cysteine into pyruvate; PGG also inhibits the formation of pyruvate, but L-cysteine, not able to bind the active site, becomes substrate for another binding site, giving cystine as different product (Figure 51 A-C).
Figure 40 - CBS time course. Real-time monitoring of CBS forward reaction by $^{13}$C filtered $^1$H NMR. Representative first (blue) and last (red) spectra are shown. Spectra showing CBS conversion of L-cysteine to L-cystathionine. The first spectrum was obtained ~2 min after the addition of enzyme, and subsequently 59 spectra were collected over a 300 min period.
Figure 41 - CSE time course. Real-time monitoring of CSE forward reaction by $^{13}$C filtered $^1$H NMR. Representative first (blue) and last (red) spectra are shown. Spectra showing CSE conversion of cysteine to pyruvate. The first spectrum was obtained ~2 min after the addition of enzyme, and subsequently 59 spectra were collected over a 300 min period.
**Figure 42 - CBS time course with compound IIa.** Real-time monitoring of CBS forward reaction by $^{13}$C filtered $^1$H NMR. Representative first (blue) and last (red) spectra are shown. Spectra showing CBS time course in the presence of compound IIa. The first spectrum was obtained ~2 min after the addition of enzyme, and subsequently 59 spectra were collected over a 300 min period.
Figure 43 - CSE time course with compound IIa. Real-time monitoring of CBS forward reaction by $^{13}$C filtered $^1$H NMR. Representative first (blue) and last (red) spectra are shown. Spectra showing CBS time course in the presence of compound IIa. The first spectrum was obtained ~2 min after the addition of enzyme, and subsequently 59 spectra were collected over a 300 min period.
Figure 44 - CSE time course with the inhibitor D,L-propargylglycine. Real-time monitoring of CSE forward reaction by $^{13}$C filtered $^1$H NMR. Representative first (blue) and last (red) spectra are shown. Spectra showing CSE inhibition with D,L-propargylglycine. The first spectrum was obtained ~2 min after the addition of enzyme, and subsequently 59 spectra were collected over a 300 min period.
Figure 45 - CSE time course. 2D $^1$H,$^{13}$C-HSQC spectra acquired before (blue peaks) and after (red peaks) the enzyme reaction.
Figure 46 - CSE time course with compound IIa. 2D $^1$H,$^{13}$C-HSQC spectrum acquired at the end of the reaction in the presence of compound IIa.
Figure 47 - CSE time course with the inhibitor D,L-propargylglycine. 2D $^{1}$H,$^{13}$C-HSQC spectrum acquired at the end of the reaction in the presence of PGG.
Figure 48 - 3D graphics of CBS time course. A. CBS time course; B. CBS time course in the presence of compound IIa.
Figure 49 - 3D graphics of CSE time course. A. CSE time course; B. CSE time course in the presence of compound IIa.
Figure 50 - 3D graphics of CSE time course. A. CSE time course in the presence of compound IIa; B. CSE time course with L-Cysteine excess.
Figure 51 - 3D graphics of CSE time course. A. CSE time course; B. CSE time course in the presence of PGG; C. Different prospective of CSE time course in the presence of PGG.
6. Conclusion

In the last few decades, investigation of the pathophysiological and pharmacological roles of hydrogen sulfide has represented a challenging research field, which is still widely unexplored.

It is noteworthy to highlight that H$_2$S relevance is constantly growing within scientific environment and it is now accepted as a novel gasotransmitter.

Many researchers are trying to address biological functions of this gaseous mediator but achievements in this field are still lacking of “proof-of-concept”, despite the large body of evidence available in the literature.

Several H$_2$S donors have been used for basic research and many drug candidates are in development. Among them, there are agents that either directly release H$_2$S when in solution (NaHS, Na$_2$S, Lawesson's reagent, GYY4137) or function as a precursor for endogenous H$_2$S synthesis (N-acetylcysteine, L-cysteine).

Currently, NaHS is the prototypical example of a H$_2$S-generating agent: it is most widely used H$_2$S donor for experimental purposes. However, this salt is not suitable for clinical purposes, as the quick release of H$_2$S may cause adverse effects, such as a rapid and excessive lowering of blood pressure.

Therefore, the design, synthesis and characterization of novel H$_2$S donors could enormously boost this research field.

In this work, we identified an innovative scaffold for the non-enzymatic release of hydrogen sulphide, acting with a slow release kinetic to emulate which is realized in a physiological context.
Specifically, a small library of 1,2,4 thiadiazolidine 3,5 diones (THIA I-VII, table 4) was prepared through suitable synthetic routes and evaluated for their H₂S-releasing properties.

These compounds, exhibiting the kinetic profile of H₂S-releasing agents, may be viewed as both powerful tools for basic studies and new potential pharmacotherapeutic agents for treatment of various diseases.

Another main line of research deals with the synthesis of selective inhibitors. At the present stage the research in this field is impaired by the lack of pharmacological tools such as selective enzymatic inhibitors. In fact, these established inhibitors exhibit low potency, low selectivity and poor cell-membrane permeability.

As such, we aimed to develop more specific and potent inhibitors of CBS and CSE towards H₂S production.

Therefore, in order to develop compounds that selectively regulate enzymatic activity, we preliminarily selected and tested commercially available cysteine surrogates. These molecules were modified with propargyl and n-butyl group and the synthetic compounds, obtained in our laboratory, were tested on rat aortic rings.

The compound showing maximal inhibitory effects in this test was an oxothiazolidine derivative, dubbed compound IIa. The effects of this compound on the enzyme kinetics was further tested on the purified enzymes using a metabolomic approach based on nuclear magnetic resonance techniques.

These studies clearly showed that compound IIa is a potent enzyme inhibitor of CSE, without affecting the CBS kinetics. Comparing its inhibitory
activity with that of the known selective CSE inhibitor, D,L-propargylglycine (PGG), a significant difference was revealed: when PGG was used, the enzyme was inhibited from producing pyruvate, ammonia and hydrogen sulphide, but surprisingly, it was still active and gave a new product, using cysteine as substrate. In contrast, the compound IIa inhibited the enzyme, keeping the concentration of substrate unaffected.

This finding suggests the possibility of using lower doses of compound IIa compared to PGG, consequently reducing all possible adverse effects.

The identification of this highly selective CSE inhibitor may help to better define the role of CSE vs CBS in the pathophysiology of the diseases where a role for the H₂S pathway has been proposed.

Furthermore, the development of such agents, particularly water-soluble, will allow us to evaluate the cross-talk of H₂S pathways with other relevant pathways (e.g., NO, COX).
7. Bibliografia


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