Selectivity of commonly used pharmacological inhibitors for cystathionine β synthase (CBS) and cystathionine γ lyase (CSE)

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BACKGROUND AND PURPOSE
Hydrogen sulfide (H2S) is a signalling molecule that belongs to the gasotransmitter family. Two major sources for endogenous enzymatic production of H2S are cystathionine β synthase (CBS) and cystathionine γ lyase (CSE). In the present study, we examined the selectivity of commonly used pharmacological inhibitors of H2S biosynthesis towards CSE and CBS.

EXPERIMENTAL APPROACH
To address this question, human CSE or CBS enzymes were expressed and purified from Escherichia coli as fusion proteins with GSH-S-transferase. After purification, the activity of the recombinant enzymes was tested using the methylene blue method.

KEY RESULTS
ß-cyanoalanine (BCA) was more potent in inhibiting CSE than propargylglycine (PAG) (IC50 14 ± 0.2 µM vs. 40 ± 8 µM respectively). Similar to PAG, L-aminoethoxyvinylglycine (AVG) only inhibited CSE, but did so at much lower concentrations. On the other hand, aminooxycetic acid (AOAA), a frequently used CBS inhibitor, was more potent in inhibiting CSE compared with BCA and PAG (IC50 1.1 ± 0.1 µM); the IC50 for AOAA for inhibiting CBS was 8.5 ± 0.7 µM. In line with our biochemical observations, relaxation to L-cysteine was blocked by AOAA in aortic rings that lacked CBS expression. Trifluoroalanine and hydroxylamine, two compounds that have also been used to block H2S biosynthesis, blocked the activity of CBS and CSE. Trifluoroalanine had a fourfold lower IC50 for CBS versus CSE, while hydroxylamine was 60-fold more selective against CSE.

CONCLUSIONS AND IMPLICATIONS
In conclusion, although PAG, AVG and BCA exhibit selectivity in inhibiting CSE versus CBS, no selective pharmacological CBS inhibitor is currently available.

Abbreviations
AOAA, aminooxycetic acid; AVG, aminoethoxyvinylglycine; BCA, ß-cyano-L-alanine; CBS, cystathionine ß-synthase; CSE, cystathionine γ-lyase; DEA/NO, diethylamine NONOate; GST, GSH-S-transferase; H2S, hydrogen sulfide; IPTG, isopropyl-b-D-thiogalactopyranoside; L-cys, L-cysteine; DL-PAG, propargylglycine; PLP, pyridoxal-5′-phosphate; δ-ALA, δ-aminolevulinic acid
Introduction

Hydrogen sulfide was first noticed in the biomedical literature as a poisonous, colourless, flammable gas with the characteristic foul odour of rotten eggs (Kimura, 2010; Wang, 2012). It can be derived from the bacterial breakdown of organic matter that occurs in swamps and sewers under anoxic conditions. It is also detected in volcanic gases, natural gas, and well waters, and is also produced in manmade activities (Szabo, 2007). More recently, it was discovered that H\textsubscript{2}S production is not restricted to bacteria, but higher eukaryotes, including humans, produce small amounts of H\textsubscript{2}S and use it as a signalling molecule (Li et al., 2011; Paul and Snyder, 2012; Wang, 2012). The biological roles of endogenous H\textsubscript{2}S are multiple and rapidly expanding. Hydrogen sulfide exerts multiple effects in most organ systems including the central and peripheral nervous systems, the cardiovascular, gastrointestinal and respiratory systems; H\textsubscript{2}S also participates in the regulation of cellular metabolism and immunological/inflammatory responses (Szabo, 2007; Lavu et al., 2010; Whiteman et al., 2010; Whiteman and Winyard, 2011; Wang, 2012).

Much of the enzymatically generated H\textsubscript{2}S is derived from two pyridoxal-5\textsubscript{-}phosphate (PLP)-dependent enzymes responsible for the metabolism of L-cysteine (L-cys): cystathionine \(\beta\)-synthase (CBS) and cystathionine \(\gamma\)-lyase (CSE) (Szabo, 2007; Wang, 2012); a third pathway that catalyses the production of H\textsubscript{2}S from L-cys via the combined action of 3-mercaptopropionate sulffurtransferase and cysteine amiotransferase has also been described (Kimura, 2010). CBS and CSE catalyse several H\textsubscript{2}S-generating reactions using cysteine and/or homocysteine as substrates (Chiku et al., 2009; Singh et al., 2009; Kabil and Banerjee, 2010; Wang, 2012). CBS is the predominant H\textsubscript{2}S-generating enzyme in the brain and nervous system and is highly expressed in liver and kidney, while CSE is mainly expressed in the liver and in vascular and non-vascular smooth muscle (Szabo, 2007; Whiteman and Winyard, 2011; Wang, 2012).

To study the role of this novel gasotransmitter, investigators have relied on genetic models, knock-down of H\textsubscript{2}S-producing enzymes by siRNA and on pharmacological inhibitors. As genetic and siRNA approaches require additional expertise and access to specialized facilities, most researchers rely on pharmacological inhibitors. The most commonly used agents to inhibit H\textsubscript{2}S biosynthesis include propargylglycine (PAG), \(\beta\)-cyanoalanine (BCA), aminooxyacetic acid (AOAA), trifluoroalanine and hydroxylamine (HA) (Szabo, 2007; Whiteman et al., 2011; Wang, 2012). PAG and BCA are claimed to be specific inhibitors of CSE, while AOAA is often used as a selective CBS inhibitor; however, their selectivity towards these two H\textsubscript{2}S-producing enzymes has not been rigorously tested. In the present study, we set out to determine whether selective inhibitors for CBS and CSE truly exist and to evaluate if studies employing the currently available pharmacological tools can reach reliable conclusions regarding the origin of H\textsubscript{2}S in biological responses.

Methods

Materials

L-cys, D-cysteine, homocysteine, PLP, \(\delta\)-aminolevulinic acid (\(\delta\)-ALA), GSH, protease inhibitor cocktail, Coomassie blue R-250, zinc acetate (ZnAc), trichloroacetic acid (TCA), N\(_2\)N-dimethyl-\(p\)-phenylenediamine-sulfate, iron(III) chloride (FeCl\(_3\)), phenylephrine (PE), sodium hydrogen sulfide (NaSH), DL-PAG, \(\beta\)-cyano-L-alanine (BCA), HA, AOAA, methionylcysteine, isoniazid, hydralazine, trifluoroalanine, amni-toxoxyvinylglycine (AVG) and pargyline were obtained from Sigma-Aldrich (Taufkirchen, Germany). D-cycloserine was obtained from ABCR GmbH & Co KG (Karlsruhe, Germany). Escherichia coli BL21 (DE3) Codon Plus cells were obtained from Stratagene. Luria-Bertani (LB) broth medium and agar were purchased from Fischer Scientific (Loughborough, UK). GSTrap FF columns were obtained from GE Healthcare (Uppsala, Sweden). Isopropyl-b-D-thiogalactopyranoside (IPTG), TritonX-100, DTT, tetramethylthioglycolinated, ammonium persulfate and amphicillin were obtained from Applichem Biochemica (Darmstadt, Germany). PBS, tris/glycine/SDS buffer (TG), Tris–HCl, PVDF membranes and DC protein assay kit were obtained from Biorad (Hercules, CA, USA). RIPA, NuPAGE LDS sample buffer and NuPAGE sample-reducing agent were purchased from Invitrogen (Carlsbad, CA, USA); Starting Block T20 blocking buffer and chemiluminescent substrate were purchased from Thermo Scientific (BioAnalytica S.A, Athens, Greece). CBS antibody was obtained from Abnova (Aachen, Germany) and CSE antibody was purchased from ProteinTech (Herford, Germany). Secondary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA).

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 \(\mu\)g·mL\(^{-1}\)).

Protein expression and purification

The expression and purification of CSE and CBS was performed as described previously with modifications (Frank et al., 2008; Huang et al., 2009). Briefly, BL21(DE3) Codon Plus cells containing either the expression vector pGEX-4T3/GST-CSE or pGEX-Kg/GST-CBS were grown at 37°C and 180 r.p.m. in LB broth medium containing 100 \(\mu\)g·mL\(^{-1}\) ampicillin to an absorption of 0.6–0.8 at 600 nm. In addition, 0.3 mM \(\delta\)-ALA was added to the culture containing pGEX-Kg/GST-CBS. Then protein expression was induced by addition of 0.1 mM IPTG. Cells containing pGEX-4T3/GST-CSE were incubated overnight at 18°C, whereas cells containing pGEX-Kg/GST-CBS were incubated overnight at 30°C. Culture was then centrifuged at 4°C and 5000x \(g\) for 10 min and the cell pellet was resuspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HP0\(_4\), 1.8 mM KH\(_2\)PO\(_4\), pH 7.8) and stored at −20°C overnight. After thawing, the suspension was sonicated in lysis buffer containing PBS and protease inhibitor cocktail for GST-CSE and PBS, 5 mM DTT, 1% Triton X-100, 100 \(\mu\)M PLP and protease inhibitor cocktail for GST-CBS. After centrifugation at 4°C for 30 min, the soluble fraction containing either the GST-CSE or the GST-CBS recombinant protein was loaded onto a GSTrap FF 1 mL affinity column previously equilibrated with binding buffer PBS. The column
was consecutively washed with five column volumes of binding buffer. Proteins attached to the column, including GST-CSE or GST-CBS recombinant proteins, were eluted with five column volumes of elution buffer (50 mM Tris–HCl, 10 mM reduced GSH, pH 8.0) and then dialysed and concentrated in 10 mM sodium phosphate buffer pH 8.2 and DTT 1 mM. The purity of the recombinant enzymes was checked by SDS-PAGE on 12% polyacrylamide gels after staining of protein bands with Coomassie Blue R-250. Protein concentration was determined using the DC protein assay kit.

**Measurement of H2S production (methylene blue assay)**

H2S determination was performed according to Stipanuk and Beck (1982) with some modifications. In the case of the CSE enzyme, each test consisted of a 100 μL reaction mixture containing 5 μg of the purified CSE enzyme, 0.01 mM PLP, 1 mM L-cys and 50 mM sodium phosphate buffer pH 8.2. For the CBS enzyme, the reaction mixture contained the same as for the CSE plus 1 mM homocysteine. The inhibitors were added to the reaction 15 min before L-cys was added to the solution. Reaction was initiated by transferring the Eppendorf tubes from ice to a 37°C shaking water bath. After 60 min of incubation at 37°C, unless otherwise indicated, the reaction was terminated by adding 1% ZnAc to trap H2S followed by 10% TCA to precipitate proteins. Subsequently, N,N-dimethyl-p-phenylenediamine-sulfate in 7.2 M HCl was immediately followed by addition of FeCl3 in 1.2 M HCl. The absorbance of the resulting solution was measured at 655 nm. H2S content was calculated against a calibration curve of standard H2S solutions. 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 et al., 2010).

**Isolated rat aortic ring assay**

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Sprague–Dawley rats (~300 g) were housed in an air-conditioned environment (22 ± 1°C, 50 ± 5% relative humidity, 12 h light-dark cycle) with free access to food and water. The animals were allowed at least 5 days to acclimatize. Animals were killed by exsanguination under deep anaesthesia by ketamine/xylazine (the femoral vein was excised). Death was ensured by opening the chest. Thoracic aortic rings (2–3 mm in length) were cut and placed in organ baths (5 mL) filled with oxygenated (95% O2 to 5% CO2) Krebs–Henseleit solution at 37°C, mounted to isometric force transducers and connected to a PowerLab data acquisition system. Aortic rings were allowed to equilibrate under a resting tension of 1 g and subsequently contracted with PE (1 μM). Once a plateau was reached, cumulative concentration-response curves to D-cysteine or L-cys were performed. In some experiments, aortic rings were incubated with AOAA (1 mM) or PAG (3 mM) 20 min before PE challenge. The experiments were repeated on at least four aortic rings, each from different rats. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010; McGrath et al., 2010).

**Western blotting**

Tissues were homogenized in RIPA in the presence of proteases inhibitors. Protein extracts were then diluted in NuPAGE LDS Sample Buffer (Invitrogen), reduced by NuPAGE sample-reducing agent and denatured at 70°C for 10 min. Samples were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with Starting Block T20 blocking buffer and then probed overnight with the indicated primary antibodies. Following incubation with an appropriate secondary antibody, immunoreactive proteins were detected using a chemiluminescent substrate.

**Statistical analysis**

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. GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used for all the statistical analysis.

**Results**

**Purification of CSE and CBS and activity optimization for H2S measurement**

Human CSE and CBS proteins were expressed in a heterologous system using *E. coli*. Recombinant proteins were then purified from bacterial lysates by affinity chromatography on GSH columns; SDS-PAGE revealed that both proteins were recovered in a highly purified form (Figure 1A,B). Moreover, the absorption spectrum of human CBS exhibited a peak at 430 nm suggesting the presence of haem in the recombinant protein (Figure 1C).

We next tested the catalytic activity of purified CSE and CBS by measuring their ability to produce H2S. Production of H2S was proportional to the amount of enzyme used (Figure 2A). As 5 μg of enzyme was the smallest amount that led to H2S synthesis that was well above the detection limit, all subsequent experiments were performed using this amount of protein. Also, all reactions were allowed to proceed for 60 min, as during the first 60 min H2S levels generated were maximal giving us optimal signal to noise readings, while reactions were still in the linear phase (Figure 2B). We then determined the amount of PLP, a cofactor for both CBS and CSE that is needed for H2S generation. Results in Figure 2C showed that 0.01 mM PLP is sufficient to produce maximal amounts of H2S from CSE; CBS isolated probably had enough PLP bound as the bacterial lysis buffer contained PLP. Both CSE and CBS generated H2S in the presence of L-cys (Figure 2D,E). However, in the presence of homocysteine, CBS produced fourfold greater amounts of H2S. In agreement to our finding, Singh et al. observed that
human CBS can generate H$_2$S from cysteine, albeit with lower $k_{cat}$ values than from cysteine and homocysteine (Singh et al., 2009). Therefore, as CBS preferentially catalyses the reaction with both homocysteine and cysteine as substrates, this reaction was used to evaluate the inhibitor compounds. Human CSE did not generate detectable H$_2$S levels under the conditions employed when homocysteine was used as a substrate (data not shown).

**Effect of inhibitors on CSE and CBS activity**
Initially, we tested the effect of several commercially available inhibitors of H$_2$S synthesis on CSE. Our results show that BCA is a more potent CSE inhibitor than PAG with IC$_{50}$ of 14 and 40 $\mu$M respectively (Table 1 and Figure 3A, B). Surprisingly, AOAA, a frequently used ‘selective’ CBS inhibitor, also inhibited CSE with an IC$_{50}$ of 1.1 $\mu$M (Figure 3D). In addition, trifluoroalanine and HA also blocked CSE, with the former being less potent than the latter (Figure 3C and E; Table 1). The most potent inhibitor of all, among the ones tested, was L-AVG (Figure 3F).

Subsequently, the ability of the above-mentioned inhibitors on CBS was tested (Figure 4). Neither PAG nor AVG had any effect on CBS activity up to the concentration used (10 and 1 mM respectively; Figure 4A, F). However, BCA reduced CBS activity when added in excess of 1 mM (Figure 4B).

**Table 1**
IC$_{50}$ values of commonly used H$_2$S inhibitors for CSE and CBS

| Compounds    | CSE, IC$_{50}$ ($\mu$M) | CBS, IC$_{50}$ ($\mu$M) |
|--------------|--------------------------|--------------------------|
| PAG          | 40.0 ± 8.0                | –                        |
| BCA          | 14.0 ± 0.16               | –                        |
| HA           | 4.83 ± 0.31               | 278.0 ± 22.0             |
| AOAA         | 1.09 ± 0.12               | 8.52 ± 0.71              |
| Trifluoroalanine | 289.0 ± 7.0           | 66.0 ± 9.0               |
| AVG          | 1.0 ± 0.1                 | –                        |

**Figure 1**
Purification of recombinant CSE and CBS. Representative SDS-PAGE of fractions after different purification steps of recombinant GST-CSE (A) and GST-CBS (B) from bacterial cell lysates. Lanes: M, protein marker; 1(A) cell lysate before purification; 1(B) PBS after GSTrap column calibration; 2(A) PBS after GSTrap column calibration; 2(B) cell lysate before purification; (3) cell lysate after purification; (4, 5) first and second wash with binding buffer respectively; (6) eluted GST-CSE or GST-CBS; (7) elution buffer. (C) UV absorption spectra of CBS in 10 mM phosphate buffer pH = 8.2. The peak at 430 nm indicates the presence of haem in CBS.
AOAA did inhibit CBS, but the IC\textsubscript{50} for this enzyme was higher than the IC\textsubscript{50} for CSE (Figure 4D). Trifluoroalanine and HA also reduced CBS activity, with trifluoroalanine being fourfold more potent than HA (Figure 4C,E).

**Effect of NO on CSE and CBS activity**

HA is known to release NO (Gross, 1985). Thus, we tested whether the inhibitory activity of this molecule on CBS and CSE might be indirect, resulting from NO release. Initially, we tested the ability of HA to alter the colour development and absorbance of NaSH; these experiments showed that HA does not interfere with colour development in the methylene blue assay (data not shown). Similarly, diethylamine NONOate (DEA/NO), a NO donor, had no effect on the NaSH standard curve when used up to 100 μM. DEA/NO concentration dependently attenuated CSE activity but did not affect CBS activity (Figure 5).

**Effect of PLP-based inhibitors on CSE and CBS activity**

We next tested the ability of several compounds that are clinically used and have some resemblance to the commonly used CSE/CBS inhibitors to reduce H\textsubscript{2}S production by the two enzymes studied. D-cycloserine and isoniazid, two antibiotics used against *Mycobacterium tuberculosis* infections, are known to inhibit multiple PLP-dependent enzymes (Riccardi et al., 2009). Both compounds showed a tendency to reduce CSE activity at 1 mM (Table 2) that for isoniazid in the case of CSE reached statistical significance. Parsalmide, an agent with anti-inflammatory, analgesic, muscle-relaxing and anxiolytic activity (Bianchetti et al., 1982), and pargyline, an antidepressant (Goldberg, 1964), which both carry C≡C bonds like PAG, failed to significantly reduce CSE or CBS activity (Table 2). Similarly, methylselenocysteine did not inhibit the activity of neither CBS nor CSE, suggesting that it cannot be used to replace the substrate L-cys from the catalytic centre of CSE and CBS.

**Effect of AOAA on H\textsubscript{2}S vasorelaxation in isolated rat aorta**

We sought to determine whether AOAA, which we found to inhibit CSE activity in vitro, would also inhibit CSE-mediated responses in tissues. To this end, we used rat aortic rings that are devoid of CBS expression (Figure 6A). Exposure of rat...
aorta to the CSE substrate L-cys, but not D-cysteine, led to a concentration-dependent relaxation (Figure 6B). Pretreatment with the CSE inhibitor PAG blocked the L-cys-driven vasodilation. In addition, pretreatment with AOAA (in a tissue that lacks CBS) blocked L-cys-induced relaxations to the same extent as PAG, while the combined treatment of PAG and AOAA offered no greater inhibition of relaxation (Figure 6C), suggesting that AOAA also inhibits CSE in a cellular milieu.

**Discussion**

The best studied inhibitor of CSE is PAG (also known as PGG), which has been co-crystallized (Sun et al., 2009) with CSE in the presence of the cofactor PLP. PAG is an antibiotic produced by *Streptomyces* sp. that irreversibly inhibits CSE through a ‘suicidal’ inactivation (Wang, 2012). Co-crystallization of CSE with PAG unveiled that PAG once bound obstructs, via steric hindrance, the accessibility of the
substrate to the active site of the enzyme by occupying the space of the side chain of the substrate. Furthermore, PAG covalently binds and traps residue Tyr114, which is believed to facilitate the release of substrate (Clausen et al., 1998; Sun et al., 2009). To inhibit CSE, PAG is typically used at concentrations in the 1–10 mM range (Whiteman et al., 2011). In our studies, we observed that the IC_{50} of PAG for CSE was 40 μM. It should be noted that while DL-PAG was used in our experiments, only the L-isoform inhibits CSE; the D-isoform is metabolized and contributes to the toxicity of the compound in vivo (Konno et al., 2000). In a frequently cited manuscript by Mok et al. (2004), an IC_{50} of 55 μM for PAG was reported in rat liver preparations; however, the racemic form of PAG (DL- or L-) used was not disclosed in this study. In contrast to the μM IC_{50} values reported with purified enzyme or cell lysates, PAG, when used in functional assays such as inhibition of L-cys-induced dilation, requires mM concentrations to inhibit 50% of the response observed (Bucci et al., 2009; 2010). Similar to what has been reported in the literature, we found that 5 mM of PAG was needed to inhibit L-cys-induced relaxation; this could perhaps be attributed to limited cell membrane permeability of PAG (Szabo, 2007). In additional experiments, we observed that PAG did not inhibit the activity of recombinant human CBS, suggesting that it can indeed be used as a selective inhibitor for CSE versus CBS. However, although selective towards CSE, PAG is not devoid of drawbacks. When used at millimolar concentrations, which are needed to fully block CSE, it also inhibits other enzymes such as aspartate aminotransferase (Tanase and Morino, 1976) and alanine aminotransferase (Burnett et al., 1992).

Figure 4
Inhibition of H_{2}S production from purified CBS by different inhibitors. Each reaction was performed in the presence of 100 μL sodium phosphate buffer containing 5 μg of CBS, 10 μM PLP, 1 mM of L-cys and 1 mM homocysteine as substrates. Inhibitors (A–F) were added 15 min before the substrates at the indicated concentration and reactions allowed to proceed for 60 min. Data are presented as mean ± SEM; n = 9; *P < 0.05 versus control.

Figure 5
Effect of DEA/NO on CSE and CBS activity. Reactions were performed as detailed in Figures 3 and 4 in the presence of the NO donor DEA/NO. Data are presented as mean ± SEM; n = 9; *P < 0.05 versus control.
-cyano-L-alanine is a neurotoxic agent found in poisonous legumes responsible for outbreaks of human neurolathyrism (Ressler, 1962; Ressler et al., 1964). BCA, unlike PAG, is a reversible inhibitor of CSE; and acts by modifying the apoenzyme transiently (Whiteman et al., 2011). We found that the IC50 for BCA is lower than PAG. In line with our observations, BCA in most cases has been shown to have lower IC50 when compared with PAG in the same preparation side by side, suggesting that BCA is a more potent inhibitor than PAG (Mok et al., 2004; Tang et al., 2005; Papapetropoulos et al., 2009). However, BCA also inhibited CBS when used above 1 mM. As this concentration is frequently used by investigators in in vitro experiments, when high BCA concentrations are used results should be interpreted with caution and not taken to automatically prove CSE involvement. In addition, much like PAG, BCA has been shown to inhibit additional enzymes such as aspartate β-decarboxylase and alanine aminotransferase (Alston et al., 1980; Cornell et al., 1984).

AOAA is a general inhibitor for aminotransferases and was proposed to also inhibit CBS (Rej, 1977). However, many studies have used it claiming it to be a CBS selective inhibitor (d’Emmanuele di Villa Bianca et al., 2009; Roy et al., 2011). To test whether AOAA is truly selective towards CBS, we tested this compound using recombinant CBS and CSE. We observed that AOAA inhibited both enzymes, with the latter being inhibited at lower AOAA concentrations. Moreover, to determine whether inhibition of CSE by AOAA also occurs at the tissue level, we used rat aortic rings. In spite of the absence of detectable CBS, AOAA inhibited the vasodilatory responses triggered by L-cys, suggesting that like in biochemical assays, AOAA is an effective CSE inhibitor and should no longer be used to selectively inhibit CBS in vitro and in vivo.

Trifluoroalanine has been shown to irreversibly inhibit a number of PLP-dependent enzymes that catalyse β- or γ-elimination reactions (Silverman and Abeles, 1976; Singh et al., 2009). Among the enzymes known to be inhibited are CSE, CBS and cystathionine γ synthase, the latter two are members of the transsulfuration pathway that exists in plants and bacteria (Steegborn et al., 1999). It has been proposed that the inactivation is due to covalent modification of the enzyme (Silverman and Abeles, 1977; Steegborn et al., 1999). Herein we observed that trifluoroalanine inhibited both CBS and CSE, and exhibited a fourfold selectivity for CBS. Another inhibitor that has been less frequently used to block H2S generation is AVG. AVG was discovered in a fermentation broth of an unidentified Streptomyces sp. as an amino acid antimetabolite that inhibited the growth of Streptomyces cellulosae (Pruess et al., 1974). AVG was previously reported to inhibit some of the enzymes of the transsulfuration pathway (Clausen et al., 1997). We observed that the IC50 of AVG for CSE is 1 μM, making AVG the most potent available inhibitor for this enzyme. Interestingly, AVG also failed to reduce CBS activity when used up to 1 mM, suggesting that in addition to being a potent CSE inhibitor, it is also the most selective for CSE. It should however be emphasized that in spite of its great selectivity for CSE over CBS, AVG has well-documented effects on other PLP-dependent enzymes. These effects should therefore be thoroughly considered when assessing the role of this compound in cell culture, tissue baths and in vivo.

HA is a product of cellular metabolism and a putative intermediate in the conversion of L-arginine to NOHA (DeMaster et al., 1989). HA has reducing and antioxidant properties and has the ability to release the gaseous media-

Figure 6
AOAA inhibits CSE biological activity in aortic tissue. (A) Representative Western blots depicting expression of CSE and CBS in rat tissues. (B,C) Phenylephrine contracted aortic rings were exposed to D- or L-cys in the absence (B) or presence (C) of inhibitors H2S synthesis, inhibitors AOAA and PAG (5 mM each). Data are presented as mean ± SEM; n = 5; *P < 0.05 versus control.
tor NO and form NO− (nitroxyln ions) (Gross, 1985). HA
is used as a general inhibitor of many haem-containing
enzymes, including CBS, although some non-haem mole-
cular targets of HA have also been observed (Spooren and
Evelo, 1997). In our study, we observed that it exhibits a
60-fold selectivity towards CSE, but it also inhibits CBS at
higher concentrations.

In higher, but not lower, eukaryotes CBS carries a haem
group that is bound to its N-terminus making CBS the only
known PLP-containing haemprotein (Wang, 2012). The func-
tion of the haem on CBS is not entirely clear. This prosthetic
group is not necessary for the catalytic action of CBS, as
removal of haem lowers but does not abolish the activity of
human CBS (Evande et al., 2004). It was recently proposed
that haem might have a structural role, facilitating folding
and promoting protein stability (Majtan et al., 2008). Binding
of NO or CO to the haem moiety has been proposed to
regulate enzymatic activity (Taoka and Banerjee, 2001).
Although both CO and NO can inhibit CBS activity, NO
binds 200-fold less tightly than CO. As HA liberates NO, we
sought to determine whether the inhibition of CBS by HA
was a direct one or was due to the NO produced. To this end,
we used the NO donor DEA/NO. We observed that CBS was
resistant at concentrations of DEA/NO up to 250 μM. In
line with this finding, Eto and Kimura (2002) reported
that neither 3-morpholinosydnonimine nor S-nitroso-N-
acetylpenicillamine up to 300 μM affects CBS activity. Since
HA already inhibits approximately 30% of CBS at 100 μM, we
conclude that the inhibition of CBS by HA is direct, rather
than through the release of NO. In addition, DEA/NO did not
inhibit CSE activity at 5 μM and caused a maximal 75%
inhibition at 250 μM, whereas addition of 5 μM HA inhibited
approximately 60% of CSE activity and 50 μM abolished CSE
activity, suggesting that the action of HA is direct and NO in-
dependent. Although the mechanism through which NO
inhibits CSE is unknown, it might involve nitrosylation of
critical cysteine residues in CSE; however, the NO-induced
inhibition of CSE is of pharmacological and not physiological
importance, as μM concentrations are needed to observe this
effect.

In summary, after testing a variety of frequently used
inhibitors for CSE and CBS, we have found that AVG is the
most potent and selective of all for CSE. PAG also exhibits
selectivity towards CSE, but its potency is lower than that of
AVG. BCA, although selective for CSE, also inhibits CBS when
used at high concentrations (Figure 7). In contrast, none
of the compounds tested exhibited significant selectivity
towards CBS. We conclude that design and development of
new inhibitors for each of the H2S-synthesizing enzymes is an
unmet need for the field so that the role of H2S-regulated
pathways in the development and treatment of disease can be
evaluated.

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Conflict of interest

The authors state no conflict of interest.

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