Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Data

High-throughput tandem-microwell assay identifies inhibitors of hydrogen sulfide signaling pathway

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Methods

Materials

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), hydroxylamine hydrochloride (HA) and L-Cys were purchased from Sangon (Shanghai, China), D,L-homocysteine from Tokyo Chemical Industry (Tokyo, Japan), D,L-propargylglycine (PAG) from Sigma, phosphoenolpyruvic acid monopotassium salt (PPPA) from Alfa Aesar (Ward Hill, MA), and porcine heart malate dehydrogenase (MDH) from Amresco (Solon, OH). The 384-well plates were from Corning Life Sciences (Acton, MA). The other materials were obtained from Sigma.

Compound library

The library contained 21,599 agents including 2,697 compounds obtained from the National Cancer Institute (NCI; Bethesda, MD), 1,563 US Food and Drug Administration (FDA) or Foreign Approved Drugs (FAD)-approved drugs from the Johns Hopkins Clinical Compound Library (JHCCL, Baltimore, MD; ref. 1), 14,400 compounds from Maybridge HitFinder collection (Thermo Fisher Scientific, Trevillett, UK), 2,939 compounds from an in-house library comprising a self-synthesized compound library and commercially available synthetic compounds or natural products from Specs (Delft, Netherlands), ChemDiv (San Diego, CA), ChemBridge (San Diego, CA), PI & PI Technology (Guangzhou, Guangdong, China).

Compounds used for follow-up studies

All hits generated from primary screening were re-ordered in the highest pure powder from original or commercial sources and used in the dose-dependent and kinetics studies as well as surface plasmon resonance assays. Hits from NCI library were re-ordered from NCI. JHU-10062 (thonzonium), JHU-8555 (quinaldine blue) and JHU-4913 (hydroxocobalamin) were purchased from Sigma, MBSEW03275 and MBS08407 were from Maybridge, and SP14311008 from ChemBridge.

Expression constructs

Human cDNA of CBS (GeneBank accession BC007257), CSE (GeneBank accession BC015807) and DDC (GeneBank accession BC008366) were purchased from Proteintech (Chicago, IL). To construct the expression vector, cDNA of full-length hCBS (CBS-FL), truncated hCBSΔ414-551 (CBS-413), CSE, and DDC was amplified by PCR with primer 1 and 2, primer 1 and 3, primer 4 and 5, primer 6 and 7, respectively (Table S2). The cDNA of *E.coli* phosphoenolpyruvate carboxylase (PEPC) was obtained by PCR with primer 8 and 9 (Table S2) from the genomic DNA of *E.coli* BL21 (DE3). The PCR products of CBS and CSE were treated with restriction enzymes EcoRI and XhoI, and subcloned into pGEX-KG to generate N-terminal GST-fusion proteins. Similarly, the PCR products of DDC or PEPC were inserted into His-Tag fusion plasmids pET28b with NheI and HindIII. All constructs were subsequently sequenced to verify the integrity of the cloned DNA.

Expression and purification of hCBS-FL, hCBS-413 and hCSE

The recombinant GST-tagged hCBS-FL, hCBS413 or hCSE were expressed and purified as described previously with following modifications[2-4]. The GST tags in the fusion proteins were removed by thrombin which produced an N-terminal extension including the residues Gly-Ser-Pro-Gly-Ile-Ser-Gly-Gly-Gly-Gly-Gly-Ile-Leu that preceded the initiator methionine.

E. coli BL21-CodonPlus cells with the hCBS or hCSE expression constructs were grown in LB medium containing ampicillin (50 μg/ml) at 37 °C to 0.8 OD_{600}. The recombinant protein was induced by adding isopropyl-1-thio-β-D-galactopyranoside (0.3 mM, final concentration) and the culture kept to grow overnight at 16 °C. The harvested cells were suspended in lysis [50 mM sodium phosphate (pH 7.5) containing 300 mM NaCl and 1 × protease inhibitor cocktail tablet (Roche)] and disrupted with a Cell Disruption Systems (Constant systems TS, UK). After removing cell debris by centrifugation at 12,000 × g for 30 min, the supernatant was mixed with GST agarose (Proteintech, Chicago, IL) and washed with buffer (50 mM Tris-HCl buffer containing 50 mM NaCl and 2.5 mM $CaCl_2$, pH 8.0). The N-terminal GST tag was then cleaved by incubating the agarose with thrombin for 4 h. After removing the agarose beads, the supernatant was collected to pass a Superdex 25 gel filtration column (Proteintech, Chicago, IL) using the mobile buffer (50 mM Tris-HCl buffer containing 50 mM NaCl, pH 7.5). The flow-through containing the hCBS or hCSE protein was collected, aliquoted and stored at -80 °C.

Expression and purification of hDDC and *E.coli* PEPC

DDC and PEPC were expressed and purified from a recombinant expression system described previously[5-7]. The resulting hDDC and PEPC proteins contained N-terminal 6 × His followed by a thrombin site (Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met-Ala-Ser) preceding the initiator methionine; PEPC contained an additional 6 × His tag immediately at its C-terminus. The *E. coli* BL21-CodonPlus cells expressing hDDC or PEPC were disrupted in PBS (140 mM NaCl, 2.7 mM KCl, 18.6 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) by the Cell Disruption Systems, and the suspension was loaded onto a Ni-NTA Agarose column. After sequentially washing with PBS buffer containing 5 mM or 50 mM imidazole, the column was eluted with ~8 mL elution buffer (300 mM imidazole in PBS buffer) and the collected samples were stored in −80 °C.

Optimization of CBS and CSE assay

Two sets containing various buffers and pH were used to determine the optimal pH for hCBS or hCSE in the presence of 100 μM PLP, 4 mM L-Cys and 4 mM D,L-HCys, and 500 nM hCBS-413 or 100 μM PLP, 5 mM L-Cys and 500 nM hCSE, respectively (Figure S1b). After the optimal buffer and pH have been determined [50 mM Tris-HCl (pH 8.6) for hCBS; 50 mM Hepes (pH 7.0) for hCSE; Figure S1b], the optimal concentrations of enzymes were determined by varying the concentrations of hCBS (0-1000 nM) or hCSE (0-1000 nM) under the above conditions.

The assays were performed by using Multichannel Electronic Pipettes with Equal Tip Spacing (Thermo Fisher, Hudson, NH) to add 10 μL of each compound containing

10% DMSO and 20 µL of the enzyme mix into the reation well (Figure 1). The compound was preincubated with the enzyme for 45 min before adding 20 µL of the substrate mix. The detection well was then filled with 50 µL 5,5′-dithiobis(2-nitrobenzoic acid), DTNB, before being tightly sealed with UltraClear film (Figure 1). The reaction solution was further incubated at 37 °C for additional 60 min before the absorbance at 413 nm of TNB was measured in a microplate reader. The final reaction contained DMSO or the tested compound in DMSO, 50 mM Tris-HCl (pH 8.6), 100 nM CBS-413 or CBS-FL, 100 µM PLP, 4 mM L-Cys and 4 mM D,L-HCys, in absence (for CBS-413) or presence (CBS-FL) of 200 µM *S*-adenosyl methionine. To activate hCBS-FL, 200 µM *S*-adenosyl methionine was added into the standard assay buffer, enhancing the activity ~3-fold[8].

High-throughput screening for inhibitors of hCBS

Agents (20 µM or 100 µM) were tested in 50 mM Tris-HCl, 100 µM PLP, 100 nM hCBS-413, 4 mM L-Cys and 4 mM D,L-HCys (pH 8.6; final volume 50 µL). After immediate addition of 50 µL of DTNB (300 µM in 262 mM Tris-HCl, 13 mM EDTA, final concentrations, pH 8.9) to the detection wells, the plates were tightly sealed with UltraClear film (Platemax PCR-TSfrom Axygen, Union City, CA) and incubated for 60 min without agitation at 37 °C before measuring OD 413 nm with a microplate reader (Synergy2 from BioTek, Winooski, VT). The Z' value of the screening assay was calculated from 16 negative samples (2% DMSO) and 8 positive samples (200 µM HA) as described[9]. Screen data from plates with a minimum Z' value of 0.5 were

accepted.

Design and validation of DDC activity assay

To detect the decarboxylase activity of hDDC, we adapted a highly sensitive and efficient method that had been used for measuring the decarboxylase activity of ornithine decarboxylase[10, 11]. The decarboxylase activity of hDDC was then linked to the consumption of NADH by using phosphoenolpyruvate carboxylase and malate dehydrogenase as coupling enzymes.

To 1 µL of a solution of compounds of interest at the indicated concentrations or of the negative control (DMSO) in the wells of the 384-well polypropylene plate (Corning Life Sciences, Acton, MA), were added 24.5 µL of the enzyme mix [50 mM Tris-HCl, 50 mM NaCl, 0.015% (w/v) bovine serum albumin, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 760 µM NADH, 330 nM PEPC, 100 µM PLP and 284 nM hDDC, pH 8.05; final concentrations]. Then, 24.5 µL of the substrate mix [50 mM Tris-HCl containing 50 mM NaCl, 0.015% (w/v) bovine serum albumin, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 10 mM phosphoenolpyruvic acid, 0.49 U malate dehydrogenase and 1.5 mM 3,4-dihydroxy-L-phenylalanine, pH 8.05; final concentration] was added to start the reaction. The plates were then tightly sealed with UltraClear film and incubated for 1 h at 37 °C before the absorbance of 340 nm was measured in a microplate reader.

Reversibility assay

Reversibility assays were performed by rapid dilution of enzyme-bound inhibitor with assay buffer[12]. hCBS-FL (20 μM, final concentration) was incubated with inhibitors at 200 μM or DMSO (control) for 40 min in standard assay buffer for hCBS. Samples were then diluted 200-fold in assay buffer and the activity was measured as described above.

Surface plasmon resonance assays

Surface Plasmon Resonance assays (SPR) with a BIAcore T200 (GE Healthcare, Uppsala, Sweden) were used to observe the direct interaction between inhibitors and hCBS. The SPR assay was performed in running buffer PBS-P (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl in presence of 5% DMSO, pH 7.4). Purified and tag-free CBS-FL (50 μg/mL) was immobilized onto a flow cell of a CM5 sensor chip using an amine coupling kit in 10 mM sodium acetate (pH 4.0). To measure the binding affinity of the inhibitors and CBS, the solutions of compounds were diluted to the following concentrations in running buffer (for NSC111041: 0, 1.95, 3.9, 7.8, 15.6, 31.25 and 62.5 μM; NSC67078: 0, 1.25, 0.625, 1.25, 2.5, 5, 10, 20 and 40 μM; SP14311008: 0, 7.8, 15.6, 31.25, 62.5 and 125 μM; HA: 0, 97.6, 195.3, 390.6 and 781 μM). The K_d values were determined with the BIAcore evaluation 3.1 software.

Kinetic analysis

hCBS utilizes Cys substrate with (Reaction 1, Table S3) or without HCys (Reaction 2, Table S3) to generate H_2S in the presence of the cofactor PLP[13]. NSC111041, NSC67078 and SP1431108 were kinetically characterized. The reaction rate was determined with hCBS at the indicated concentrations of inhibitors against increasing concentrations of PLP (0–15 mM in the presence of 20 mM L-Cys and 10 mM D,L-HCys substrate; Reaction 1), Cys (0–100 mM in the presence of 100 μM PLP; Reaction 2), or HCys (0–100 mM in the presence of 20 mM L-Cys and 100 μM PLP; Reaction 1). The data were fitting to the Michaelis-Menten inhibition equation for determination of the values of K_i, αKi or αKi' by using GraphPad Prism 5 (Table S1) [12, 14]. To illustrate the type of inhibition (competitive, noncompetitive or mix type inhibition), these data were analyzed via re-plots of the slopes of Lineweaver-Burk plots against varying concentrations of inhibitor (Fig. 3b and c; Fig. 6b-d; Fig. 7b-d; Fig. 8d).

Molecular modeling

Inhibitors of hCBS (NSC111041, NSC67078 and SP14311008) were docked by molecular modeling into the active site of the crystal structure of the complex of hCBS and PLP cofactor (PDB code: 1JBQ; ref. 15). The 3D structures of NSC111041, NSC67078 and SP14311008 (Table S1) were optimized by energy minimization (Discover Studio version 3.5; Accelrys, San Diego, CA) and docked into the active site of hCBS with the Discover Studio/CDOCKER module, a CHARMm-based molecular dynamics simulated-annealing program[16].

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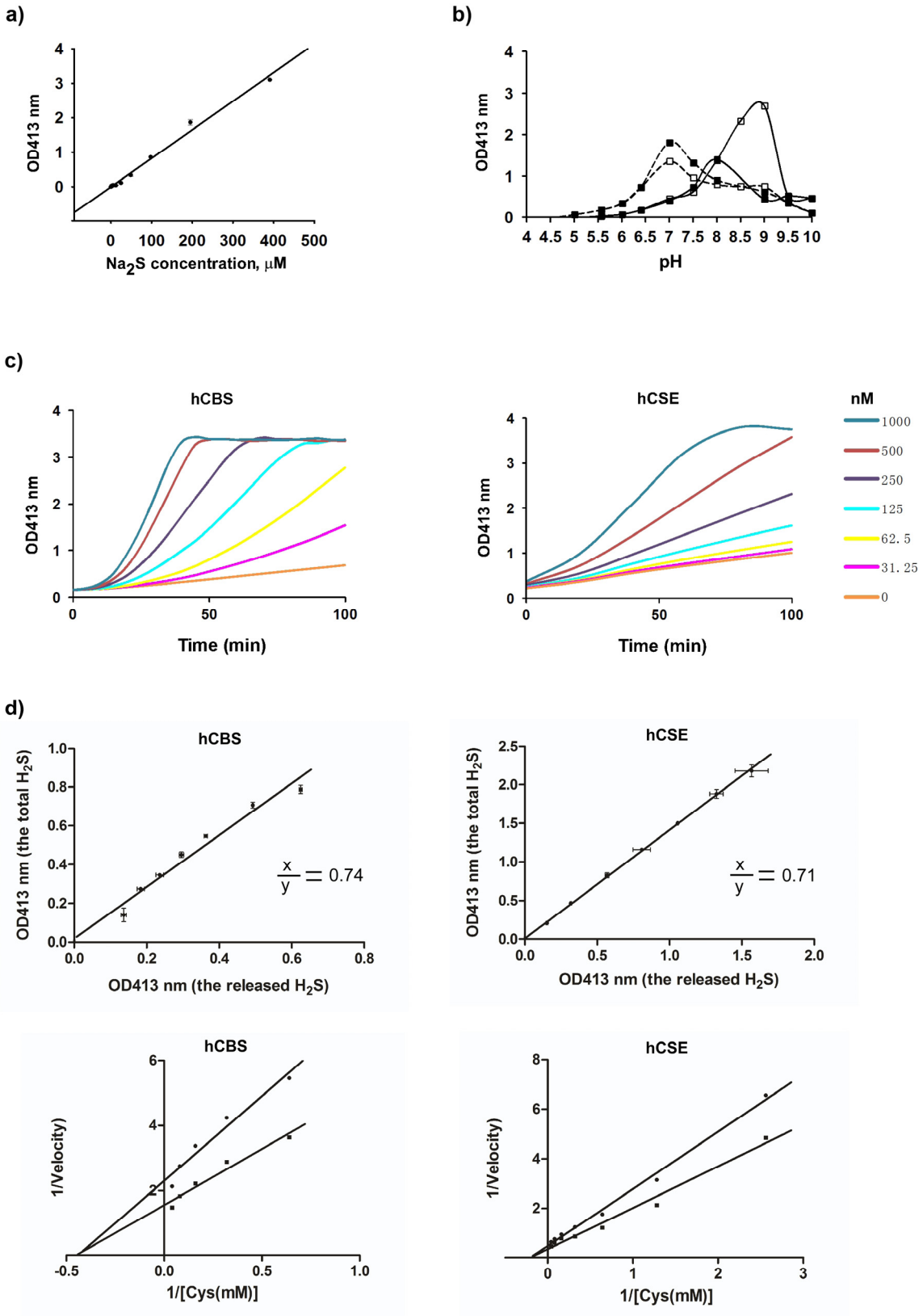

a)
OD413 nm
Na2S concentration, μM
b)
OD413 nm
pH
c)
hCBS
hCSE
nM
1000
500
250
125
62. 5
31. 25
0
OD413 nm
Time (min)
d)
hCBS
hCSE
OD413 nm (the total H2S)
OD413 nm (the released H2S)
x/y = 0.74
x/y = 0.71
1/Velocity)
1/[Cys(mM)]

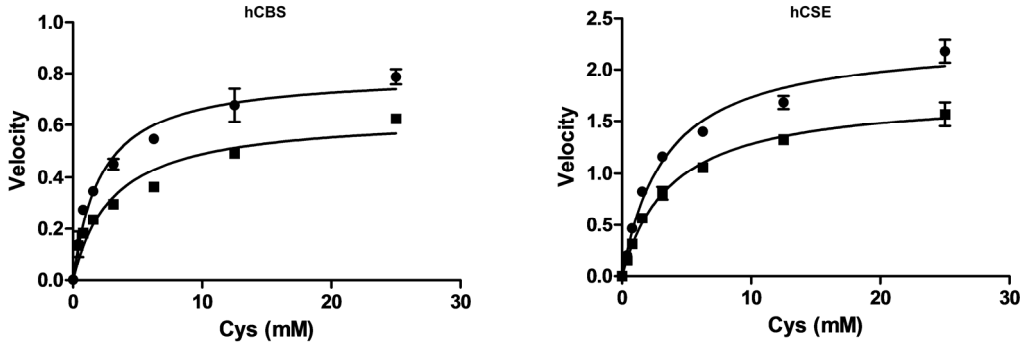


Figure S1 Optimization of hCBS or hCSE assays. (a) Standard curve of OD 413 nm of generated TNB, thiobis(2-nitrobenzoic acid), versus Na_2S concentration. Na_2S (25 µL, resulting in the indicated final concentrations) followed by 25 µL of DTNB (300 µM, final concentration) were pipetted into the detection wells of a 192-tandem-well-plate. The plates were immediately tightly sealed with UltraClear film, and the OD at 413 nm was measured in a microplate reader. The obtained data (in triplicate) are presented as means ± SD. (b) pH-Rate profile of H_2S-generating reactions catalyzed by hCBS or hCSE. The profiles of hCBS (——) and hCSE (- - -) were determined in two sets of buffers. One buffer set (□) included 100 mM sodium acetate (pH 4.5-5.5), 50 mM Bis-Tris-HCl (pH 6-7), 50 mM Tris-HCl (pH 7.5-9), and 50 mM glycine (pH 9.5-10). The other set (■) was 100 mM sodium acetate (pH 4.5-5.5), 50 mM Bis-Tris-HCl (pH 6-6.5), 50 mM Hepes (pH 7-8), and 50 mM glycine (pH 9-10). hCBS-413 or hCSE were dissolved in the respective buffers and assayed at a final concentration of 500 nM. (c) Time course of reaction as a function of enzyme concentration. Concentrations from 0 to 1000 nM of hCBS-413 or hCSE were assayed in 50 mM Tris-HCl (pH 8.6) or Hepes (pH 7.0), respectively. (d) The

calibration of the released H_2S and the total H_2S generated in the tandem well-based assay by hCBS or hCSE. Various concentrations of Cys (0, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25 or 50 mM) were used as a substrate to validate and calibrate the ratio of DTNB-absorbed H_2S and the total H_2S generated by hCBS (left pane) or hCSE (right pane) under the optimal pH, buffer and enzyme concentrations for hCBS or hCSE as determined in (c). 50 µl Cys substrate mix in present or absent of enzyme were placed into the reaction well of tandem-microwell remoulded from a 96-microwell protein crystallization plate (Corning), followed by adding 50 µl DTNB into the detection well. For measuring the total H_2S generated by hCBS or hCSE in the tandem-microwell plate, a truncated tube containing 20 µL of 4 M H_2SO_4 were hanged upside-down in the reaction well of the tandem-microwell before the plate sealed with UltraClear film. After 40 (for CBS) or 20 min (for CSE) reaction time, the plate was centrifuged at 1000 rpm for 10 sec to spin down the H_2SO_4 hanging on the wall of the upper tube into the enzymic solution (final concentration, 1.1 M; pH<0.1), which could stop the enzymic reaction and transpire the solubilized H_2S from the solution. The DTNB from the acid-treated tandem-microwell or non-treated were then transferred into the 384-microwell plate for measuring of the absorbance of OD413 nm after additional 1 h incubation to ensure the completely absorption of acid-evaporated H_2S by DTNB. The regression line was drawn by comparing the absorbance values of DTNB from the acid-treated (y-axis, in the upper panels) and non-treated tandem-microwell (x-axis, in the upper panels) at the various concentrations of Cys (from left to right: 0, 0.781, 1.563, 3.125, 6.25, 12.5, 25 or 50

mM for CBS; 0, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5 or 25 mM for CSE). The perpendicular errors reflect the SD from the values of y-axis (n=2), and horizontal error bars are from x-axis (n=2). The treatment of the H_2SO_4 to the assay solution containing only Cys substrate did not generate detectable H_2S gas. The Lineweaver-Burk plots (middle panels) were drew to compare the K_m of Cys for CBS or CSE under acid-treated or non-treated conditions followed by non-linear fitting the data to the appropriate Michaelis-Menten equation for determination of K_m values (lower panels). Lineweaver-Burk plots represent the average of duplicates. The values in non-linear global fitting plots are presented in mean ± SD (n=2).

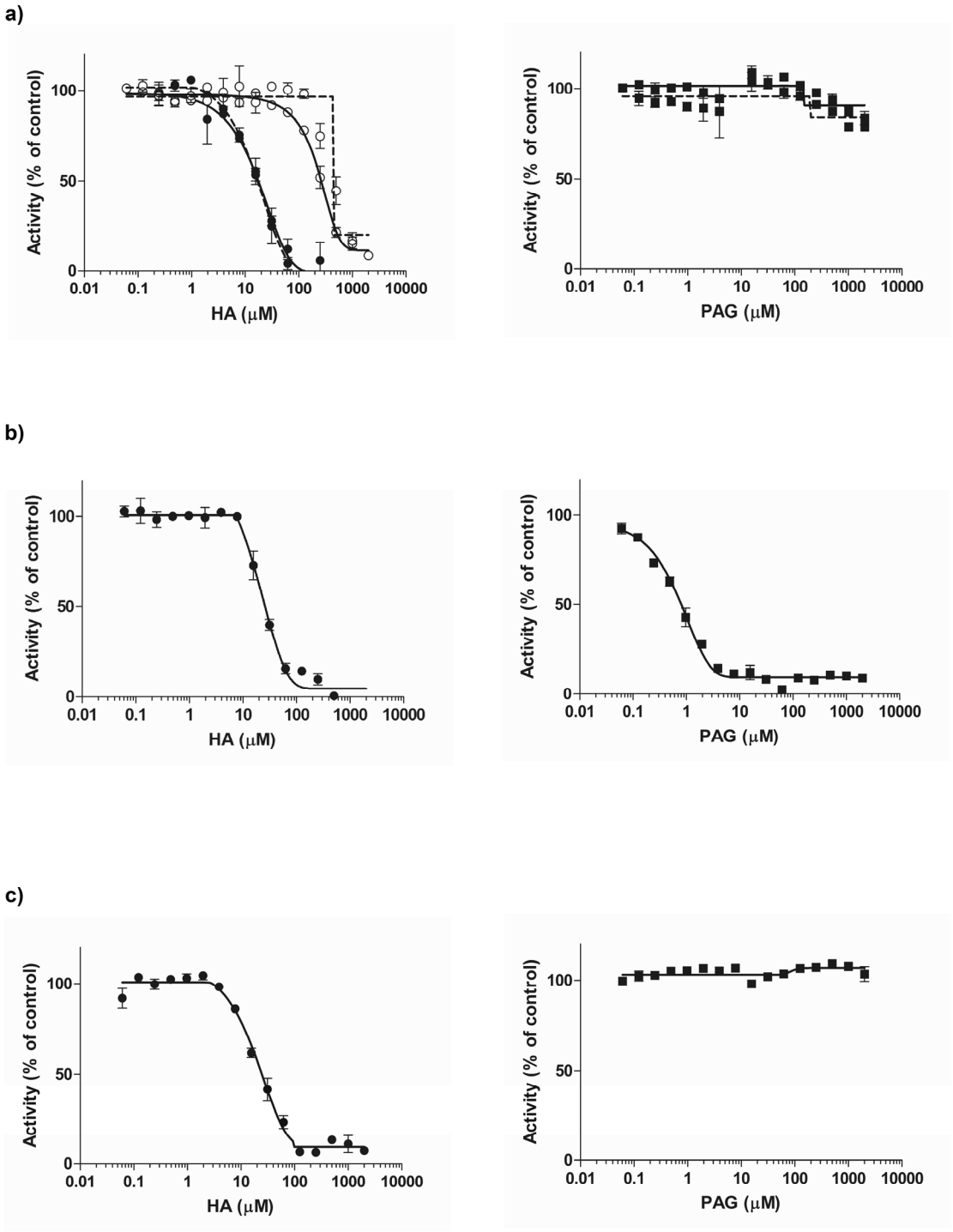


Figure S2 Dose-dependent inhibition of the activity of hCBS, hCSE or hDDC by HA and PAG. Various concentrations of HA (0-2000 μM; ●) or PAG (0-2000 μM; ■) were incubated with hCBS-413 (– –, a) or hCBS-FL (——, a), hCSE (——, b) or hDDC

(——, c), and assayed under the conditions and according to the procedures described in Methods in the Supplementary Data. The dose-dependent effects of HA to hCBS-413 and hCBS-FL were also measured in 50 mM Hepes (○, pH 7.4; refs. 13, 17) instead of Tris-HCl (pH 8.6). The data are expressed as percent of control (DMSO). Data (n=4) are presented as means ± SD. The dose-dependent curves fitted to the data points with GraphPad Prism 5.

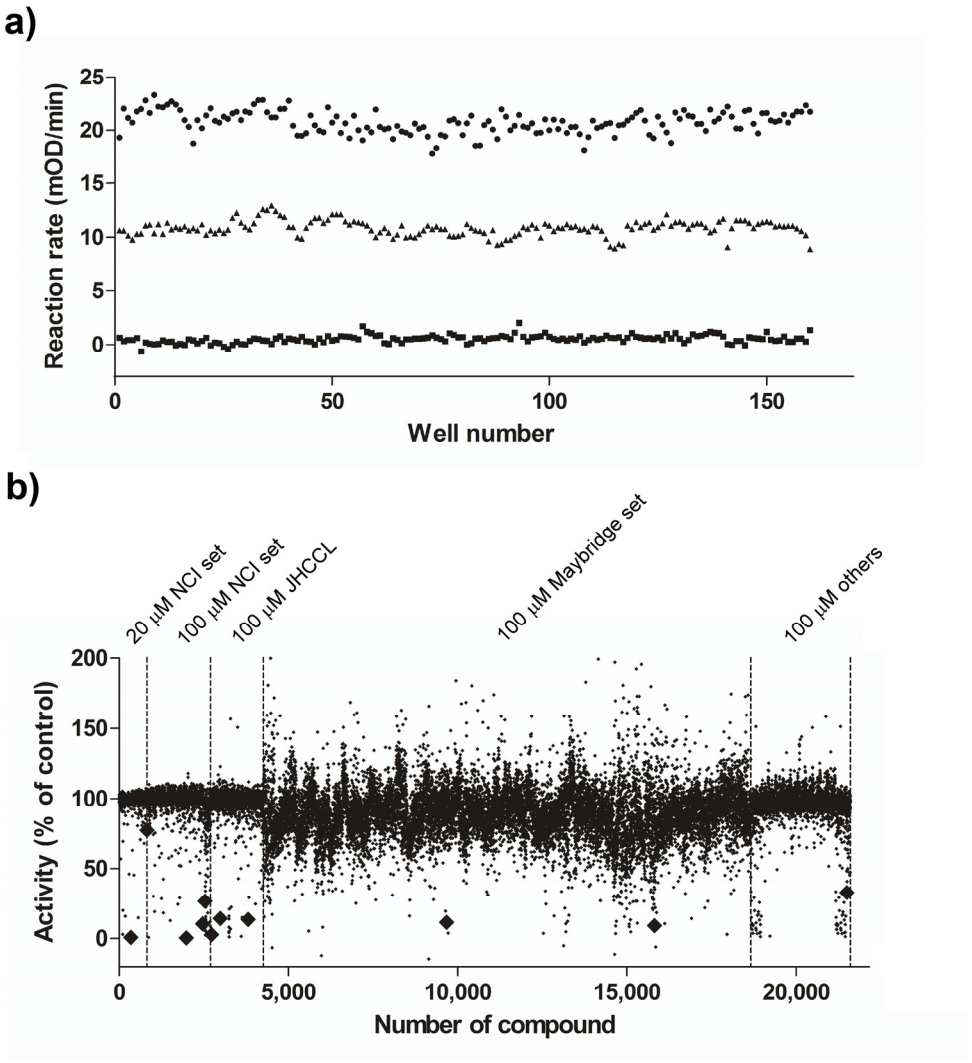


Figure S3 Validation of high-throughput assay and screening of compound libraries. (a) Well-to-well reproducibility of detection of H_2S generated by hCBS in 192-tandem-well plates (for conditions, see Methods). The absorbance data points of the top trace represent 192 wells containing the reaction buffer with 2% DMSO without inhibitor as control (●), the middle and bottom points were obtained in the presence of 20 μM (▲) and 200 μM (■) HA, respectively. (b) High-throughput screening for CBS inhibitors with 192-tandem-well plates. The library contained 879 compounds from NCI mechanistic set (tested at 20 μM) and 20720 compounds (tested

at 100 μM) from NCI, JHCCL, Maybridge Hitfinder collection or other libraries, adding up to a total 21599 compounds. The residual activities are expressed as percent of control. The eleven compounds listed in Table S1 (dose-response curves with IC_{50} <50 μM) are indicated by enlarged symbols (◆).

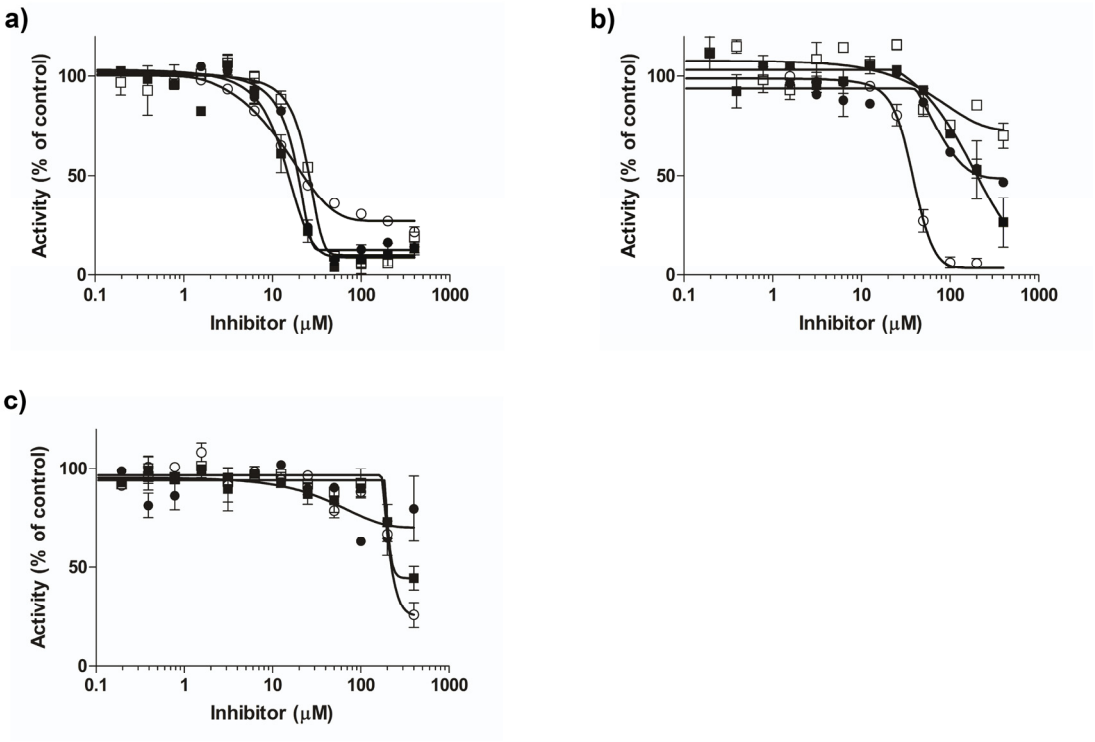


Figure S4 Effects of MBSEW03275, JHU-8555, SP14311008 and MBS08407 on the activity of hCBS, hCSE and hDDC. (a) Dose-dependent effects of MBSEW03275 (■), JHU-8555 (●), SP14311008 (○) and MBS08407 (□) on the activity of hCBS-FL. (b) Effects of the same inhibitors on the activity of hCSE. (c) Effects on the activity of hDDC. MBS08407 was only tested at the concentration from 0 to 100 μM, since the absorbance of higher concentration of it interfered with the DDC assay. The data are expressed as percent of control (DMSO). Data (n=4) are presented as means ± SD. The curves were fitted to the data points with GraphPad Prism 5.

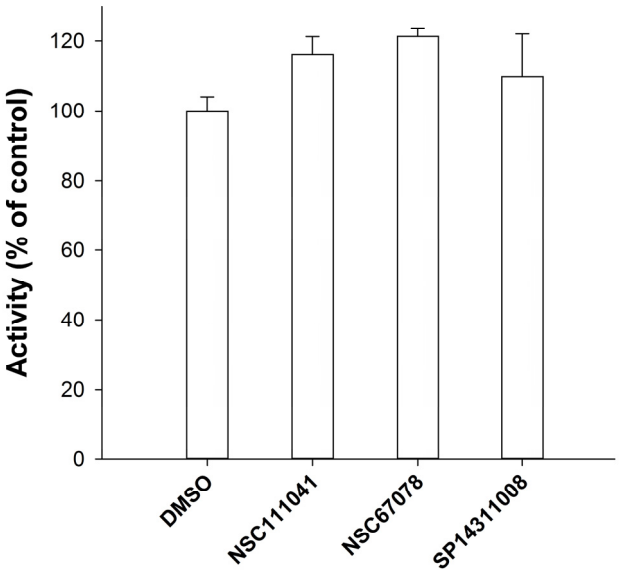


Figure S5 The inhibitors bind to hCBS reversibly. After incubation with indicated inhibitors at 200 μM for 40 min, hCBS-FL (20 μM) was diluted 200-fold into assay buffer and the activity was measured and compared with the control (DMSO). Data (n=4) are presented as means ± SD.

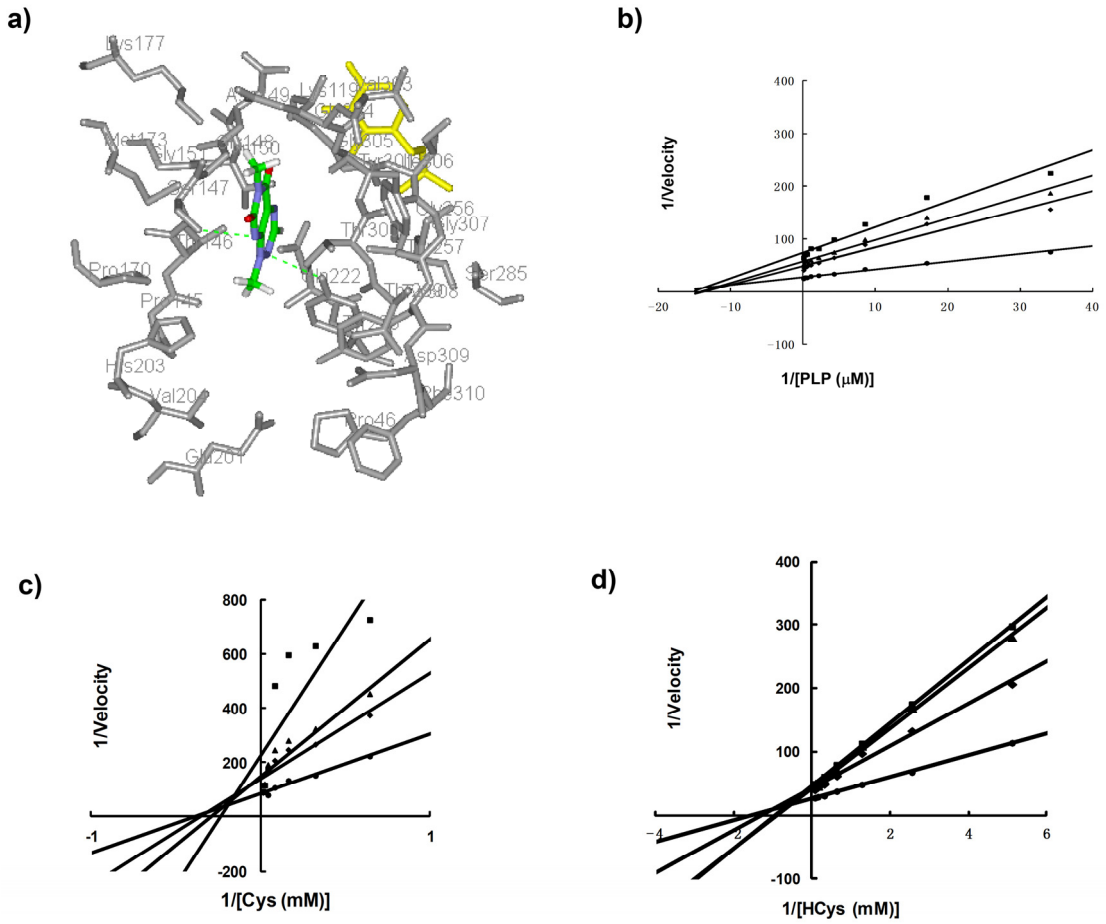


Figure S6 Molecular modeling of inhibitor NSC67078 into the active site of CBS and kinetic characteristics. (a) NSC67078 was docked into the active site of hCBS, residues surrounding the NSC67078 within a distance of 5 Å are in grey. The vitamin B_6 (PLP) cofactor is displayed in yellow and NSC67078 is in green with default atom types (O, N). The hydrogen bonds formed by NSC67078 with hCBS, were shown with green dotted lines. (b) Inhibition of hCBS-FL by NSC67078 as a function of PLP concentration with Cys (20 mM) together with HCys (10 mM) as substrates (non-competitive inhibition constant αK_i=11.7±0.7 μM). (c) Inhibition of hCBS-FL by NSC67078 as a function of Cys concentration in the absence of HCys and in the presence of 100 μM PLP (mixed inhibition constant αK_i=27.5±0.1 μM).(d) Inhibition

of hCBS-FL by NSC67078 as a function of HCys concentration in the presence of 20 mM Cys and 100 μM PLP (mixed inhibition constant αK_i'=13.0±0.3 μM). NSC67078 concentrations were ■, 25 μM; ▲, 12.5 μM; ◆, 6.25 μM; ●, control. Each point in the Lineweaver-Burk plots represents the average of duplicates. The αK_i or αK_i' values were calculated by using the non-linear fitting method for enzyme kinetics in GraphPad Prism 5.

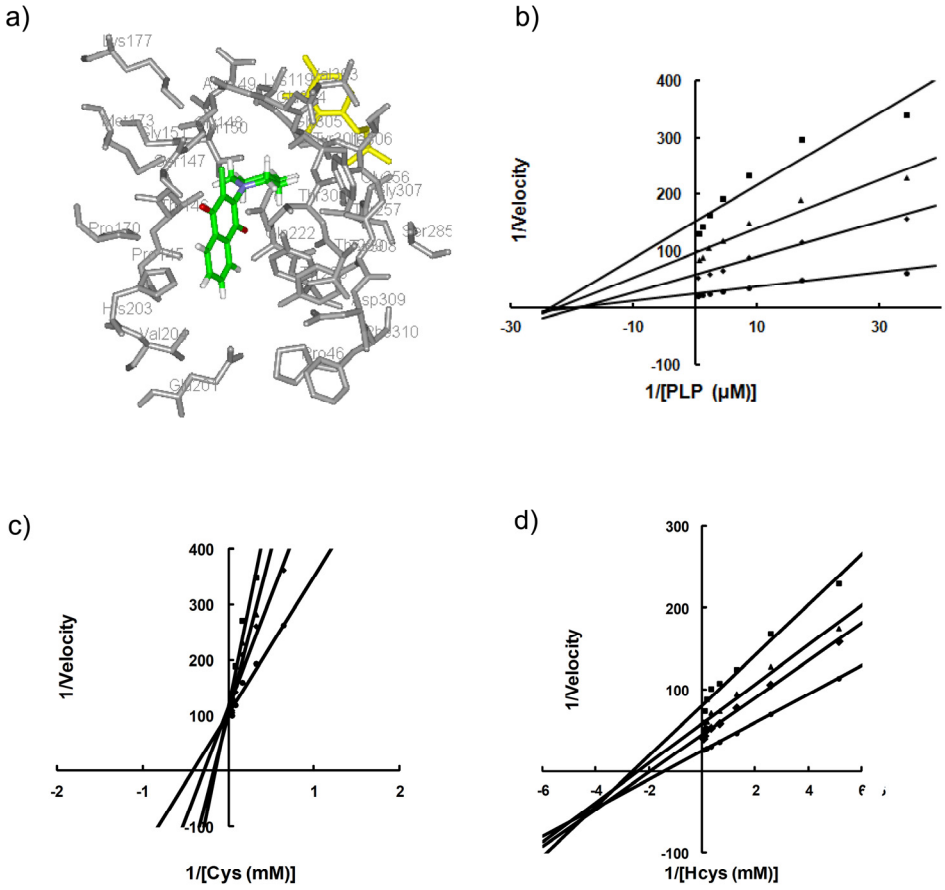


Figure S7 Molecular modeling of inhibitor SP14311008 into the active site of CBS and kinetic characteristics. (a) SP14311008 was docked into the active site of hCBS, residues surrounding the SP14311008 within a distance of 5 Å are in grey. The vitamin B_6 (PLP) cofactor is displayed in yellow and SP14311008 is in green with default atom types (O, N). (b) Inhibition of hCBS-FL by SP14311008 as a function of PLP concentration with Cys (20 mM) together with HCys (10 mM) as substrates (non-competitive inhibition constant αK_i=9.9±0.4 μM). (c) Inhibition of hCBS-FL by SP14311008 as a function of Cys concentration in the absence of HCys and in the presence of 100 μM PLP (competitive inhibition constant K_i=38.9±4.3 μM). (d) Inhibition of hCBS-FL by SP14311008 as a function of HCys concentration in the presence of 20 mM Cys and 100 μM PLP (mixed inhibition constant αK_i'=28.6±0.6

μM). SP14311008 concentrations were ■, 50 μM; ▲, 25 μM; ◆, 12.5 μM; ●, control. Each point in the Lineweaver-Burk plots represents the average of duplicates.The Ki, αK_i or αK_i' values were calculated by using the non-linear fitting method for enzyme kinetics in GraphPad Prism 5.

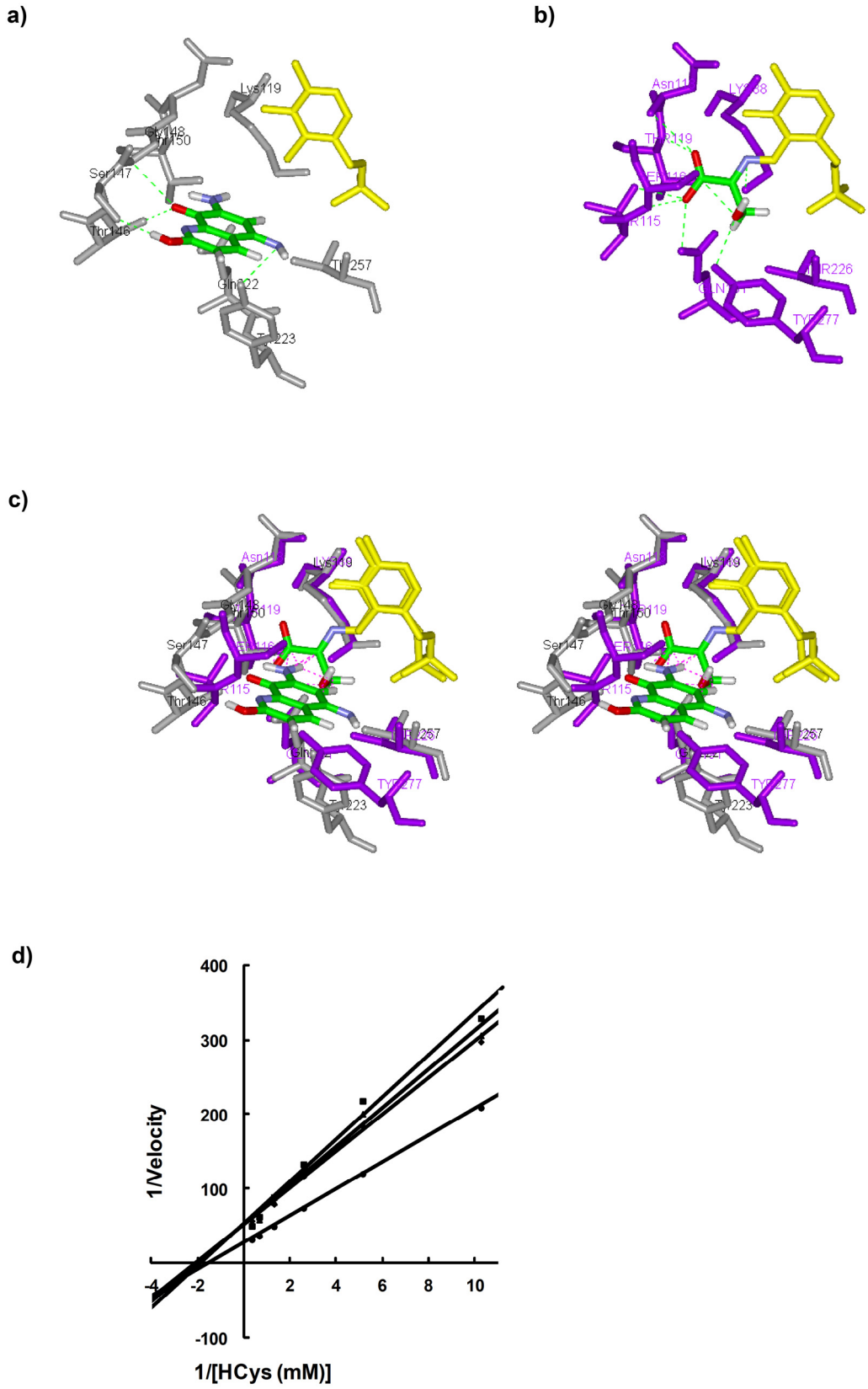


Figure S8 The binding site of NSC111041 overlaps with that of serine substrate*.

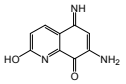
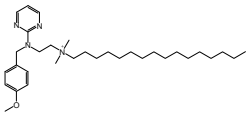
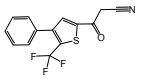
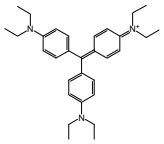
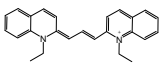
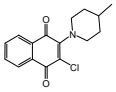
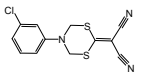
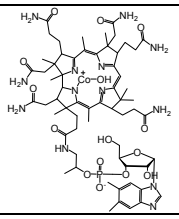
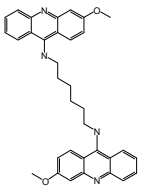
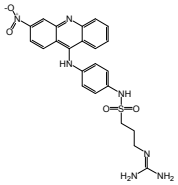
(a) NSC111041 was modelled into the active site of the crystal structure of hCBS and

PLP (PDB code 1JBQ, ref. 15). (b) The active site of dCBS together with the phosphopyridoxyl-serine (PDB code 3PC4[18]). (c) Superposed stereo view of the residues of the active site of hCBS (grey, the side chains are labeled with black fonts) and dCBS (purple, labels in purple) together with NSC111041 (colored in default atom type) and the conjugate of phosphopyridoxyl (vitamin B_6, yellow)-serine (default atom type). The superposition of hCBS and dCBS was performed on the basis of the sequence alignment of hCBS and dCBS (Fig. S5 of ref. 18) by Discovery Studio. Residues surrounding NSC111041 or phosphopyridoxyl-serine within a distance of 3 Å are indicated. The hydrogen bonds formed by NSC111041 or serine substrate with hCBS or dCBS, respectively, are shown with green dotted lines. The intramolecular bumps between NSC111041 and serine are indicated by red dotted lines. (d) Inhibition of hCBS-FL by NSC111041 as a function of HCys concentration in the presence of 20 mM Cys and 100 μM PLP (mixed inhibition constant αK_i'=18.7±0.8 μM). NSC111041 concentrations were ■, 12.5 μM of NSC111041; ▲, 6.25 μM; ◆, 3.13 μM; ●, control. Each point in the Lineweaver-Burk plots represents the average of duplicates.The αK_i' value was calculated by using the non-linear fitting method for enzyme kinetics in GraphPad Prism 5.

*In the crystal structure of drosophila CBS, the substrate serine was found to form hydrogen bonds with Thr115, Ser116, Gln191, and Tyr277 of dCBS (Figure S8b,c)[18]. The alignment of the amino acid sequences of dCBS and hCBS as well as the superposition of their 3D structures indicate that these residues are homologous with

the residues surrounding NSC111041. Superimposing the binding sites of NSC111041 in hCBS and of serine in dCBS resulted in substantial steric overlap between NSC111041 and serine (Figure S8c). Apparently, NSC111041 shares the binding region with serine.

Table S1 Chemical structures and IC_{50}, Ki, αKi, αKi' or Kd values of selected inhibitors.

Name	Compound	IC_{50} (μM); hCBS-413* or -FL†	IC_{50} (μM); hCSE	IC_{50} (μM); hDDC	Ki‡, αKi¶,Δ or αKi'*(μM) for binding to hCBS-FL	Kd (μM) for hCBS-FL
NSC111041		4±0.5*,†	2.5±0.1	35±1.7	10.3±0.9¶ 1.5±0.2‡ 18.7±0.8#	8
NSC67078		12±0.5*,†	30±2.3	≥400	11.7±0.7¶ 27.5±0.1Δ 13.0±0.3#	74
JHU-10062 or NSC5648 Thonzonium		8±0.1* 12±0.8†	25±0.1	16±1.1	n.d.	n.d.
MBSEW03275		15±2.3*,†	200±27.8	200±28.1	n.d.	n.d.
NSC8675 Ethyl violet		15±0.1*,†	15±0.2	25±1.3	n.d.	n.d.
JHU-8555 Quinaldine blue		20±0.1*,†	200±2.9	>400	n.d.	n.d.
SP14311008		20±0.1*,†	40±2.9	300±23.6	9.9±0.4¶ 38.9±4.3‡ 28.6±0.6#	82
MBS08407		15±0.5* 25±3.0†	>400	>100	n.d.	n.d.
JHU-4913 Hydroxocobalamin		35±2.8*,†	40±0.4	150±15.8	n.d.	n.d.
NSC260610		30±1.2* 40±4.9†	170±6.8	110±11.9	n.d.	n.d.
NSC177365		40±8.2* 60±6.8†	200±15.2	60±3.6	n.d.	n.d.
Hydroxylamine (HA)	$HO-NH_2$	20±1.5*,† 400±49.9*,§ 250±10.3†,§	20±0.5	20±1.8	n.d.	365

[‡]Ki, the dissociation constant for competitive inhibitors, was measured with Cys at varying concentrations as a single substrate in the presence of 100 μM PLP.

[ᴧ]αKi, the dissociation constant for the mixed-type inhibitors, was measured with Cys at varying concentrations as a single substrate in the presence of 100 μM PLP.

[¶]αKi values was measured with PLP at varying concentrations as a cosubstrate in the presence of both 20 mM Cys and 10 mM HCys.

[#]αKi' values was measured with HCys at varying concentrations and 20 mM Cys as substrate and 100 μM PLP .

[§]IC_{50} values were determined in 50 mM Hepes (pH 7.4).

Table S2 Primer sequences

No.	Primer	Usage
1	5'-AAATTTGAATTCTACCTTCTGAGACCCCCCAGGC-3'	5' primer for constructing pGEX-KG CBS-FL or CBS-413
2	5'-ATATATCTCGAGTCACTTCTGGTCCCGCTCC-3'	3' primer for constructing pGEX-KG CBS-FL
3	5'-ATATATCTCGAGTCAACGGAGGTGCCACCACCAGGGC-3'	3' primer for constructing pGEX-KG CBS-413
4	5'-AAATTTGAATTCTACAGGAAAAAGACGCCTCCTC-3'	5' primer for constructing pGEX-KG CSE
5	5'-ATATATCTCGAGCTAGCTGTGACTTCCACTTGG-3'	3' primer for constructing pGEX-KG CSE
6	5'-TATATATAGCTAGCAACGCAAGTGAATTCCGAAGGAGAGGG-3'	5' primer for constructing pET28b DDC
7	5'-AAATTTAAGCTTCTACTCCCTCTCTGCTCGCAGCACG-3'	3' primer for constructing pET28b DDC
8	5'-TATATATAGCTAGCAACGAACAATATTCCGCATTGCG-3'	5' primer for constructing pET28b PEPC
9	5'-AAATTTAAGCTTTCAGTGGTGGTGGTGGTGGTGGCCGGTATTACGCATACCTGCCGC-3'	3' primer for constructing pET28b PEPC

Table S3 The reaction for generation of H_2S catalyzed by hCBS or hCSE.

Reaction No.	Reaction for generating H_2S	Reaction catalyzed by CBS*		Reaction catalyzed by CSE‡	
		The valid reaction†	The most effective reaction at physiological conditions§	The valid reaction†	The most effective reaction at physiological conditions§
1	HCys + Cys	†	§	†	
2	Cys	†		†	§
3	Cys + Cys	†		†	
4	HCys			†	
5	HCys + HCys			†	

*Obtained from ref. 13
‡Obtained from ref. 17