

## Supplementary Information

### Discovery of Selective Cystathionine $\beta$ -Synthase Inhibitors by High-Throughput Screening with a Fluorescent Thiol Probe

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## Methods

### Materials

7-Diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM), methionine, aminooxyacetic acid (AOAA) and D,L-homocysteine were purchased from Sigma-Aldrich (Missouri, USA). HT29 cells (human colon adenocarcinoma cell line), HepG2 cells (human hepatocellular liver carcinoma cell line) and NCM356 cells (human nonmalignant colonic epithelial cell line) were purchased from ATCC (USA). Fetal bovine serum (FBS) was obtained from Biological Industries (Beit HaEmek, Israel), the penicillin-streptomycin solution (100×) was obtained from TransGen Biotech (Beijing, China), and the cell counting kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Methanethiol (CH<sub>3</sub>SH) was purchased from ANPEL Laboratory Technologies Inc. (Shanghai, China). The natural product library containing 6,491 compounds was obtained from the National Compound Resource Center (Shanghai, China). The other materials were purchased from Sangon (Shanghai, China). The 11 lead compounds obtained from secondary screening were purchased from BioBioPha (Kunming, China), J&K Scientific (Beijing, China) and TopScience (Shanghai, China). Sikokianin C, Podocarpusflavone A, 2",4"-Di-O-(Z-p-coumaroyl) afzelin, Caraphenol A and Myricetin were obtained from BioBioPha. Agathisflavone, 3'-hydroxy-volkensiflavon, cupressuflavone, NP-014428 and NP-003872 were obtained from J&K Scientific. Hypericin were purchased from TopScience. Unless otherwise specified, all chemicals were used as received.

### Expression and purification of hCBS and hCSE

The full-length human cystathionine-β-synthase (hCBS) was expressed and purified as previously described, with the following modifications.<sup>[1]</sup> Generally, cell pellets obtained from a 2-L culture were resuspended in 200 mL of phosphate-buffered saline (PBS) supplemented with 20 mg of lysozyme, 5 mg of PLP, and an “EDTA-free” protease inhibitor tablet (Roche). The cell lysate was prepared by sonication, centrifuged at 12,000 rpm for 30 min at 4 °C, and the supernatant was obtained. Subsequently, the supernatant was loaded on a GSTrap FF column (GE Healthcare, USA) that had been equilibrated with PBS. The column was subsequently washed with at least 20 column volumes of PBS, and glutathione S-transferase (GST)-fused hCBS was eluted with 20 mM glutathione (GSH) in 50 mM Tris, pH 8.0. The GST-fused hCBS was cleaved with thrombin at a final concentration of 5 U/mg protein at 4 °C, and the GST tag was removed using a Q sepharose FF column (GE Healthcare, USA) that had been equilibrated with 50

mM Tris buffer, pH 8.0. The hCBS protein was eluted with a 200 mL linear gradient of 0 to 500 mM NaCl in 50 mM Tris-HCl, pH 8.0. Fractions containing the pure hCBS protein were pooled and dialyzed, and the aliquoted enzyme was stored at - 80 °C.

The cDNA of human cystathionine- $\gamma$ -lyase (hCSE) was amplified from mRNA templates extracted from HepG2 cells and cloned into the pET22b plasmid to generate N-terminal histidine-tagged proteins. *E. coli* BL21(DE3) cells with the hCSE expression constructs were grown in LB medium containing kanamycin (30  $\mu$ g/mL) until the optical density at 600 nm ( $OD_{600}$ ) reached 0.4-0.6. The expression of hCBS was induced by adding 0.1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), and the cells were further cultured for 12 h at 25 °C. The harvested cells were resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 100 mg/L lysozyme, and an "EDTA-free" protease inhibitor tablet (Roche). The resuspended cells were then incubated on ice for 1 h and sonicated with Ultrasonic Cell Disruption System (Scientz, Ningbo, China). The supernatant was collected by centrifugation at 12000 rpm for 30 min at 4 °C and loaded on a HisTrap FF column (GE Healthcare, USA) that had been equilibrated with 20 mM imidazole and 300 mM NaCl in 50 mM Tris-HCl, pH 8.0. The recombinant hCSE was eluted with 300 mM imidazole in 50 mM Tris-HCl, pH 8.0. The collected fractions were desalted using a HiPrep desalting column (GE Healthcare, USA) that had been equilibrated with 50 mM Tris-HCl, pH 8.0. Fractions containing the pure hCSE protein were pooled, concentrated, and stored at - 80 °C.

### **CBS and CSE activity assay using the CPM fluorescent probe**

The CBS and CSE activity assays were performed in 200  $\mu$ L of reaction buffer using methylcysteine as the substrate. The reaction mixture containing 50 mM HEPES, pH 7.4, 15  $\mu$ M CPM fluorescent probe, and 1  $\mu$ g of hCBS or hCSE was added to a 96-well plate, and the reaction was started by adding 10 mM methylcysteine. The fluorescence of the mixture was monitored at 460 nm ( $\lambda_{ex}$  = 400 nm) for 10 min at room temperature. The 96-well plate was placed in a multifunctional microplate reader, which was set up to shake the plate for 15 sec prior to collecting the first data point.

The Michaelis constants ( $K_m$ ) of hCBS and hCSE were determined by varying the concentration of the methylcysteine substrate (0.1-40 mM) in the presence of 50 mM HEPES buffer, pH 7.4, 15  $\mu$ M CPM fluorescent probe, and 1  $\mu$ g of hCBS or hCSE at 25 °C. The  $K_m$  values of hCBS and hCSE were calculated using a double-reciprocal plot.

### **Measurement of CBS and CSE activity using H<sub>2</sub>S production assays**

H<sub>2</sub>S generation catalyzed by CBS using cysteine and homocysteine as substrates was measured as previously described.<sup>[2]</sup> The enzyme reaction mixture containing 50 mM Hepes, pH 7.4, 0.4 mM lead nitrate, 25 mM homocysteine and hCBS was preincubated at 37 °C for 3 min, and then, the reaction was started by adding 25 mM cysteine. An extinction coefficient of 5500 M<sup>-1</sup> cm<sup>-1</sup> at 390 nm was used to estimate the lead sulfide concentration. The method used to measure the CSE activity in the H<sub>2</sub>S production assays was identical to the CBS assay, with the exception that cysteine was used as the only substrate.

### **High-throughput screening for hCBS inhibitors**

All studies were performed in 200 µL of reaction buffer per well containing 50 mM Hepes, pH 7.4, 15 µM CPM fluorescence probe, 1 µg of hCBS and 2 µL of DMSO (dimethyl sulfoxide). The methylcysteine concentration was 10 mM and the final concentration of each library compound was 100 µM. After the addition of the substrates, the fluorescence of the reaction buffer was monitored at 460 nm ( $\lambda_{ex} = 400$  nm) for 10 min at room temperature. The microplate reader was set up to shake the 96-well plate for 15 sec prior to collecting the first data point. Additionally, the 1% DMSO reaction buffer without the library compound was used as the negative control, and the reaction buffer containing 100 µM AOAA was used as the positive control. The Z' value of the screening assay was calculated from 8 negative samples (1% DMSO) and 8 positive samples (100 µM AOAA) as described.<sup>[3]</sup> Screen data from plates with a Z' value of more than 0.5 were accepted. The library compounds that showed >80% inhibition of hCBS activity were selected for the subsequent validation.

### **Hit validation**

Compounds that were capable of reducing hCBS activity by more than 80% in the initial screen were analyzed in 200 µL of reaction buffer containing 50 mM Hepes, pH 7.4, 15 µM CPM fluorescence probe, 1 µg of hCBS and 2 µL of DMSO. The methylcysteine concentration was 10 mM and the final concentration of the selected compounds was 50 µM. After adding the substrates, the fluorescence of the reaction buffer was monitored at 460 nm ( $\lambda_{ex} = 400$  nm) for 10 min at room temperature. The 1% DMSO reaction buffer without the inhibitor was used as the negative control, and the reaction buffer containing 100 µM AOAA was

used as the positive control. The compounds that showed >50% inhibition of hCBS activity were accepted.

### **Hit validation with CH<sub>3</sub>SH**

All studies were carried out in 200  $\mu$ L of reaction buffer per well containing 50 mM Hepes, pH 7.4, 15  $\mu$ M CPM fluorescence probe and 2  $\mu$ L of DMSO. For each inhibitor, the concentration of CH<sub>3</sub>SH was held steady at 100  $\mu$ M and the concentration of compound was varied from 0-100  $\mu$ M. The microplate reader was set up to shake the 96-well plate for 15 sec prior to measuring fluorescence at room temperature.

### **Reversibility of the inhibitors**

hCBS (10  $\mu$ M) was incubated with the 11 inhibitors at a final concentration of 100  $\mu$ M or DMSO (control) for 1 h at room temperature in 50 mM Hepes, pH 7.4. Samples were then ultrafiltered using 1 mL spin column (Millipore, USA) for 5 times (dilution factor >1000 times) and the activity was measured using H<sub>2</sub>S production assay.

### **Cell culture**

HT29 cells, HepG2 cells and NCM356 cells from ATCC were cultured in DMEM supplemented with 10% FBS and a 1% penicillin-streptomycin solution at 37 °C and 5% CO<sub>2</sub>.

### **Determination of the IC<sub>50</sub> values of the inhibitors**

The dose-dependent effects of the inhibitors on both hCBS and hCSE activities were determined. The IC<sub>50</sub> values of the hCBS and hCSE inhibitors were measured in the presence of 25 mM homocysteine, 25 mM cysteine, 1  $\mu$ g of hCBS or hCSE and 0.4 mM lead nitrate in a 200  $\mu$ L total volume (5% DMSO and 50 mM Hepes, pH 7.4). The 5% DMSO reaction buffer without the potential inhibitors was used as a control. The purity of all compounds which were used to determine the IC<sub>50</sub> values is 95% or more.

### **Measurement of H<sub>2</sub>S production from HT29 and NCM356 cell lysates**

Cells were rinsed thrice with ice-cold PBS and then lysed in RIPA buffer (Solarbio, Beijing, China). After centrifugation at 12,000 g for 10 min, the supernatant was collected and the protein concentration was determined. For the measurement of H<sub>2</sub>S production, the methylene blue assay was used with some modifications [4]. The reaction mixture (200  $\mu$ L) contained 30  $\mu$ g protein of the cell lysates, 5 mM cysteine

and 5 mM homocysteine in 50 mM Tris-HCl buffer, pH 8.0. The inhibitors (1 mM AOAA or 2 mM PAG ) were added to the reaction mixture 10 min before reaction was initiated by adding 5 mM cysteine. After 30 min incubation at 37 °C, the reaction was terminated by adding 10% TCA to precipitate CBS protein.

Subsequently, *N,N*-dimethyl-*p*-phenylenediamine-sulfate in 7.2 M HCl was immediately followed by addition of FeCl<sub>3</sub> in 1.2 M HCl. The absorbance of the resulting solution was measured at 670 nm. H<sub>2</sub>S content was calculated against a calibration curve of standard H<sub>2</sub>S solutions. AOAA and PAG (DL-propargylglycine, which is a specific inhibitor of hCSE) were purchased from Sigma (Missouri, USA).

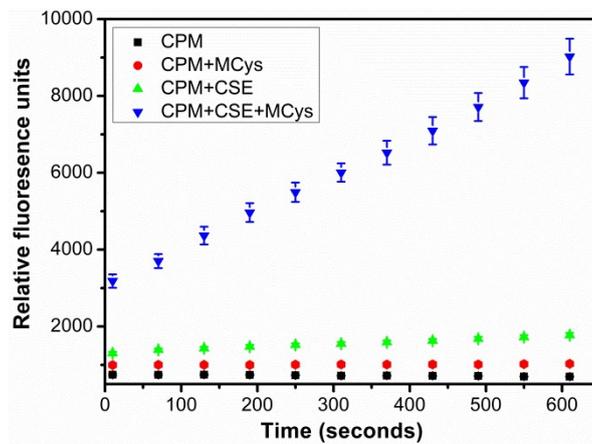
### **Determination of the IC<sub>50</sub> values of the inhibitors for HT29 cells and NCM356 cells**

The IC<sub>50</sub> values of hCBS-selective inhibitors for HT29 cells (which express high levels of hCBS) and NCM356 cells (which over-express hCSE, but only over low levels of hCBS) were determined using the CCK-8 assay. The exponentially growing HT29 cells were inoculated in a 96-well plate at a density of 1×10<sup>4</sup> cells/well. After 24 hours of culture, fresh medium containing varying concentrations of the CBS-selective inhibitors was added and the cells were cultured for an additional 24 h. The CCK-8 solution (10 µL/well) was added to the each well followed by an incubation for an additional 2 h at 37 °C. The optical density at 450 nm (OD<sub>450</sub>) was measured using a microplate reader and the medium without CBS inhibitors was used as the control group. The cell viability rate was calculated using the following formula:

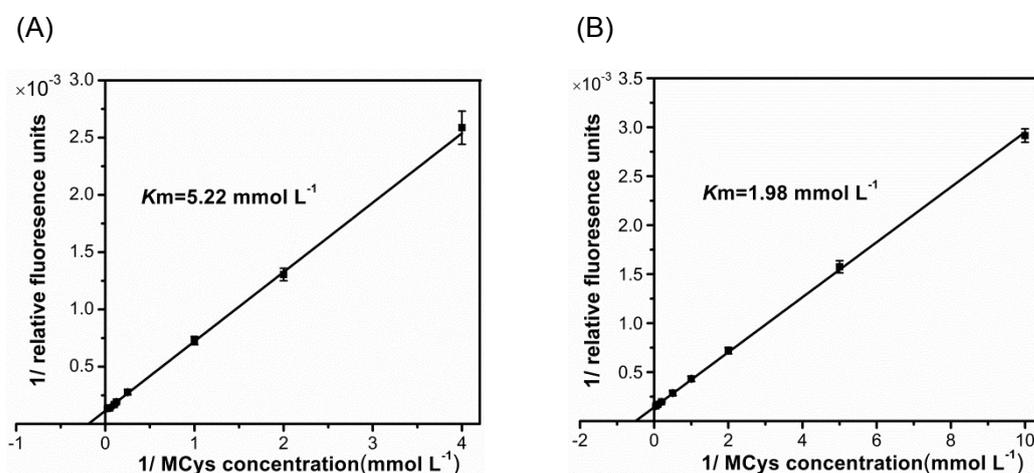
$$\text{Viability rate} = (\text{experimental group OD}_{450} / \text{control group OD}_{450}) \times 100\%$$

### **References:**

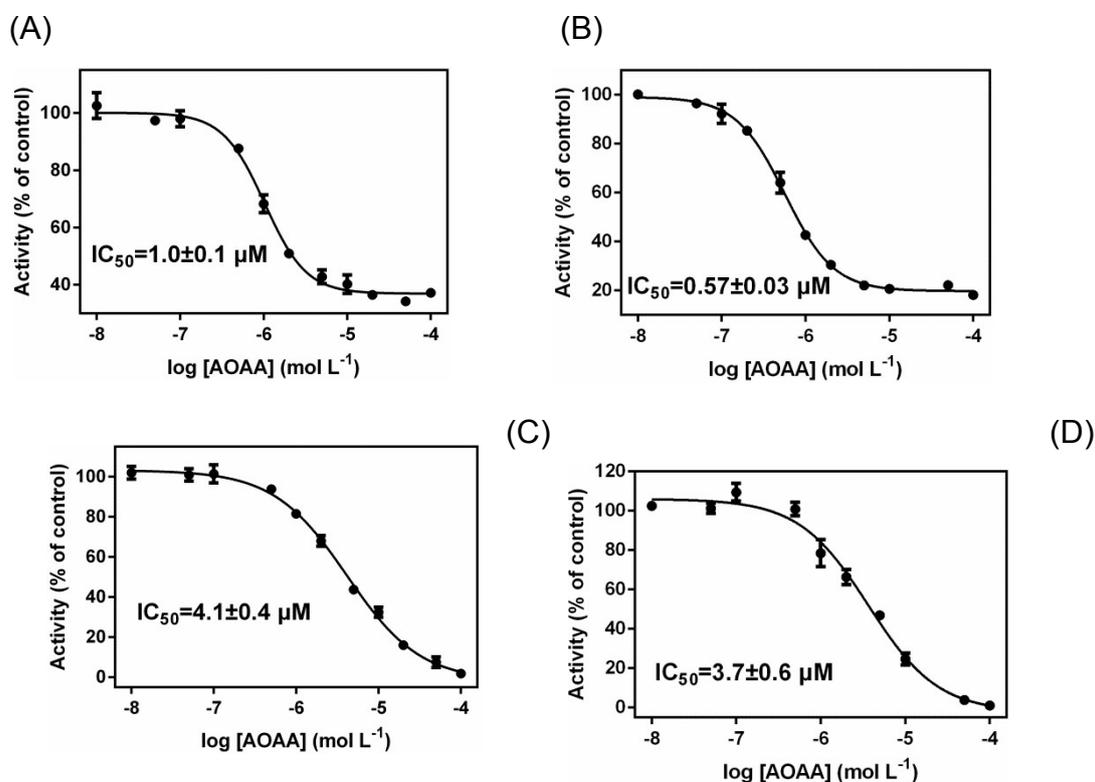
- 1 S. Taoka, S. Ohja, X. Shan, W. Kruger, R. Banerjee, *J. Biol. Chem.* 1998, **273**, 25179.
- 2 T. Chiku, D. Padovani, W. Zhu, S. Singh, V. Vitvitsky, R. Banerjee, *J. Biol. Chem.* 2009, **284**, 11601.
- 3 J. Zhang, T. Chung, K. Oldenburg, *J. Biomol. Screen*, 1999, **4**, 67.
- 4 A. Asimakopoulou, P. Panopoulos, C. Chasapis, C. Coletta, Z. Zhou, G. Cirino, A. Giannis, C. Szabo, G. Spyroulias, A. Papapetropoulos, *Br. J. Pharmacol.* 2013, **169**, 922.



**Figure S1 The activity of human cystathionine- $\gamma$ -lyase (hCSE) was monitored using the CPM fluorescent probe.** hCSE activity was monitored using 10 mM methycysteine as the substrate in the presence of 15  $\mu$ M CPM fluorescent thiol probe, 2  $\mu$ g of hCSE in a 200  $\mu$ L reaction mixture containing 50 mM Hepes, pH 7.4. The fluorescence of the reaction mixture was monitored at 460 nm ( $\lambda_{ex}$  = 400 nm) for 600 seconds. Mcys= methycysteine. CPM=7-Diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin.

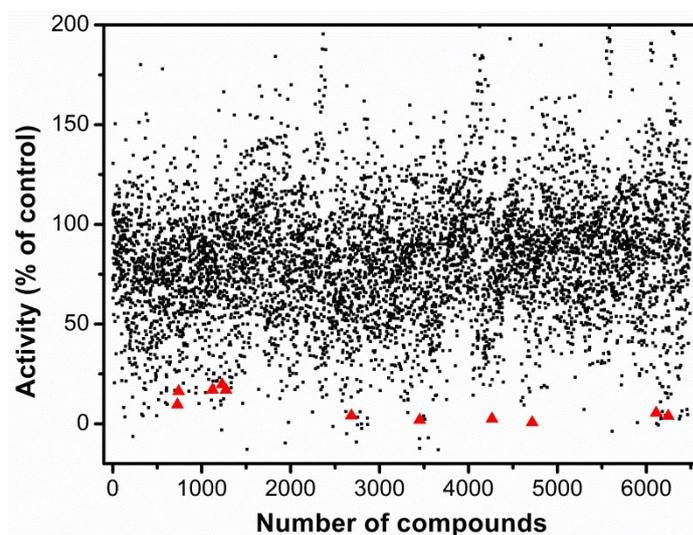


**Figure S2 Determination of the Michaelis constants ( $K_m$ ) of hCBS and hCSE using methylcysteine as the substrate.** The  $K_m$  values were calculated using a double-reciprocal plot. (A) The  $K_m$  of hCBS was determined by varying the concentration of the substrate methylcysteine (0.25-40 mM) in the presence of 50 mM Hepes buffer, pH 7.4, 15  $\mu$ M CPM fluorescence probe, and 2  $\mu$ g of hCBS in a 200  $\mu$ L reaction mixture at 25  $^{\circ}$ C. (B) The  $K_m$  of hCSE was determined by varying the concentration of the substrate methylcysteine (0.1-20 mM) in the presence of 50 mM Hepes buffer, pH 7.4, 15  $\mu$ M CPM fluorescence probe, and 2  $\mu$ g of hCSE in a 200  $\mu$ L reaction mixture at 25  $^{\circ}$ C.

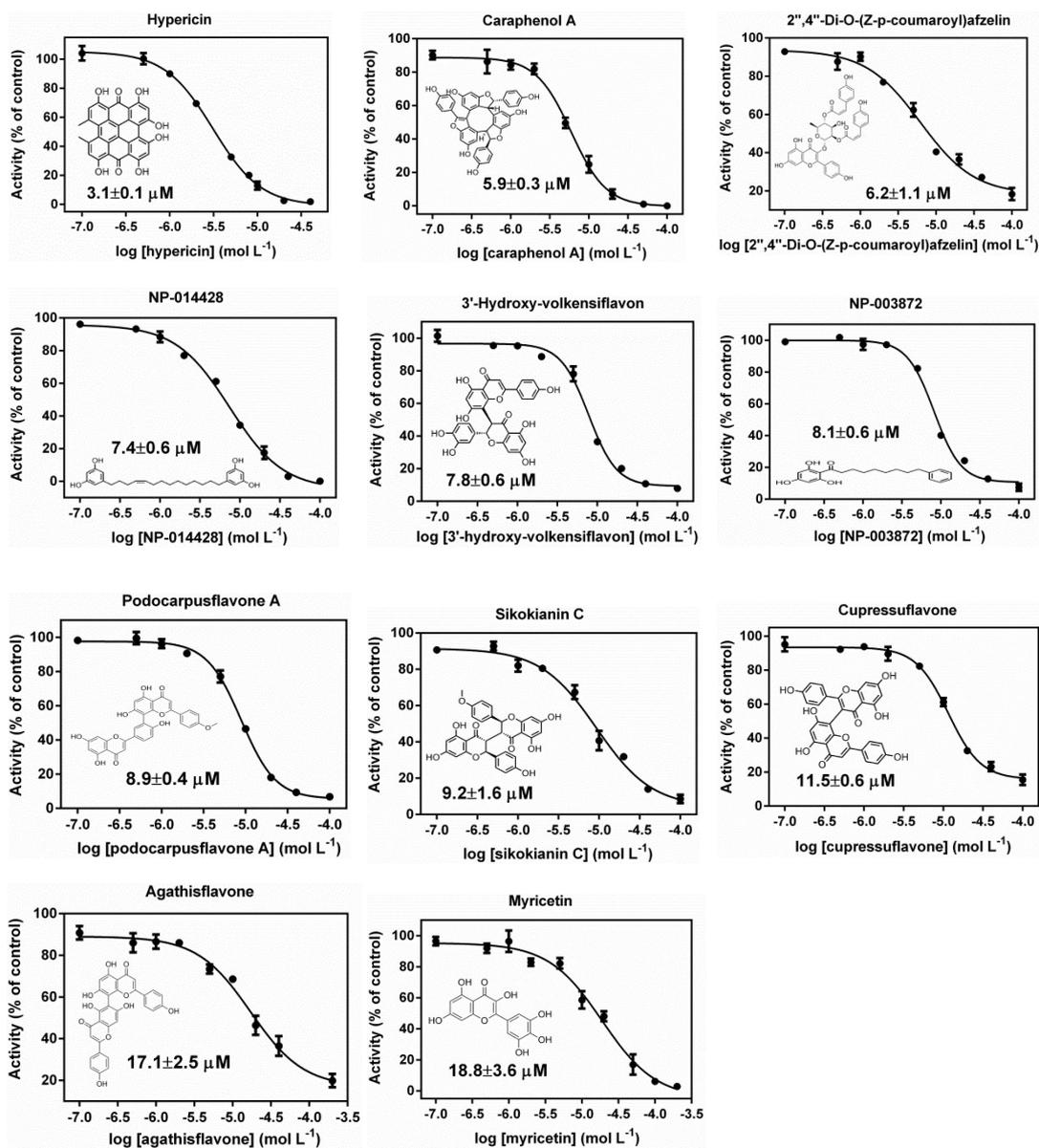


**Figure S3 Dose-dependent inhibition of hCBS and hCSE activities by AOAA in the different assays.**

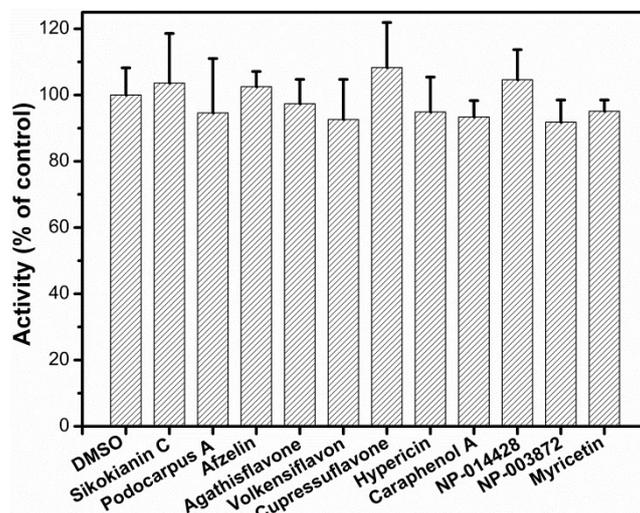
The dose-dependent effects of AOAA on hCBS (A) and hCSE (B) were measured using 10 mM methylcysteine as the substrate in the presence of various concentrations of AOAA (0-100  $\mu M$ ), 15  $\mu M$  CPM fluorescent probe, and 1  $\mu g$  of hCBS or hCSE in a 200  $\mu L$  mixture containing 50 mM Hepes, pH 7.4, according to the Methods. Simultaneously, the dose-dependent effects of AOAA on hCBS (C) and hCSE (D) were determined in the presence of various concentrations of AOAA (0-100  $\mu M$ ), 0.4 mM lead nitrate, and 1  $\mu g$  of hCBS or hCSE in a 200  $\mu L$  mixture containing 50 mM Hepes, pH 7.4, using 25 mM homocysteine and 25 mM cysteine as the substrates, according to the Methods. The reaction mixture without AOAA was used as the control group. The data represented the enzyme activity of the samples compared with the control group and were presented as the means  $\pm$  SD ( $n=3$ ). GraphPad Prism 6 software was used to fit the data.



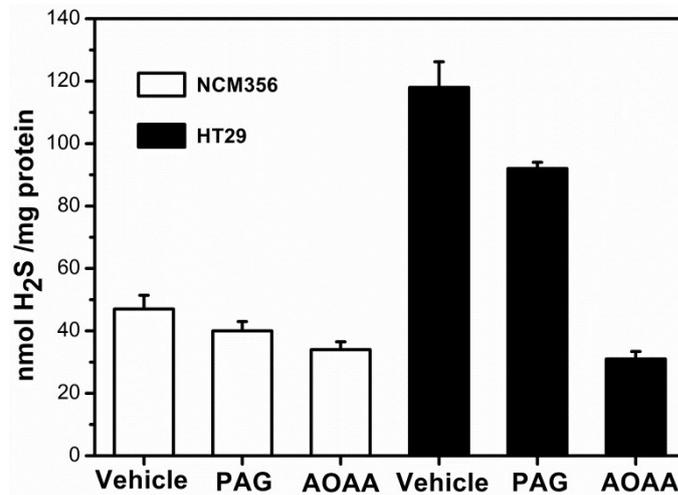
**Figure S4 High-throughput assay for screening the hCBS inhibitors.** The natural product library contained 6,491 compounds was obtained from the National Compound Resource Center (Shanghai, China). The residual activities of hCBS are expressed as a percent of the control. The eleven compounds with  $IC_{50}$  values of less than 20  $\mu$ M are indicated by red triangles.



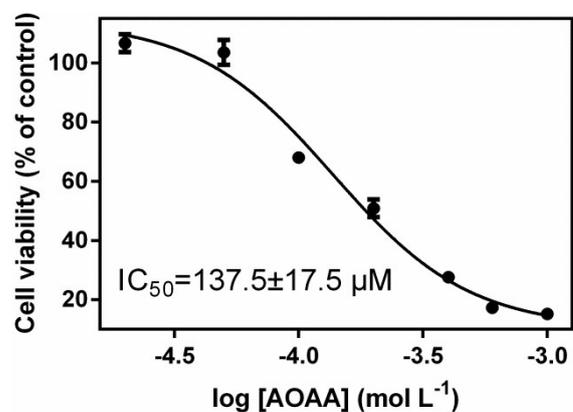
**Figure S5 Dose-response curves of the 11 compounds against hCBS.** The IC<sub>50</sub> values were determined using homocysteine and cysteine as substrates in the presence of varying concentrations of inhibitors, 0.4 mM lead nitrate A, and hCBS in a 200 μL mixture containing 50 mM Hepes, pH 7.4, according to the Methods. GraphPad Prism 6 software was used to fit the data.



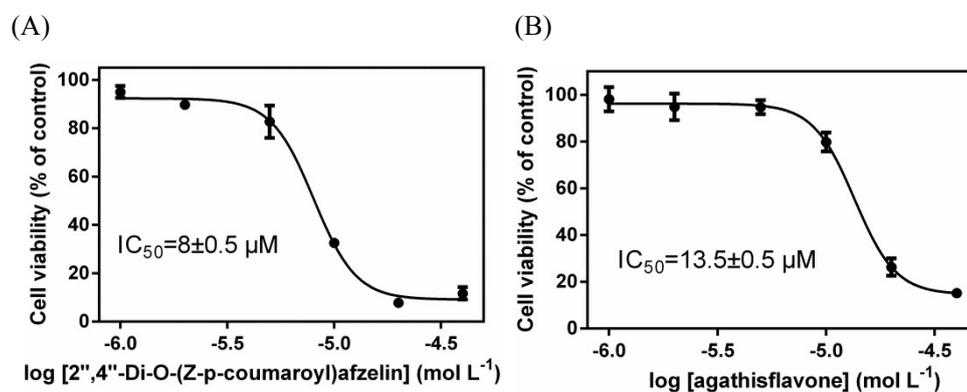
**Figure S6 The 11 hits inhibit the hCBS activity reversibly.** hCBS (10  $\mu$ M) was incubated with various inhibitors at a final concentration of 100  $\mu$ M for 1 h at room temperature in 50 mM Hepes, pH 7.4, followed by ultrafiltration using 1 mL spin column for 5 times (dilution factor >1000 times). The activity was measured using H<sub>2</sub>S production assay. The data represented the enzyme activity of the samples compared with the control group (DMSO) and were presented as the means $\pm$ SD (n=3).



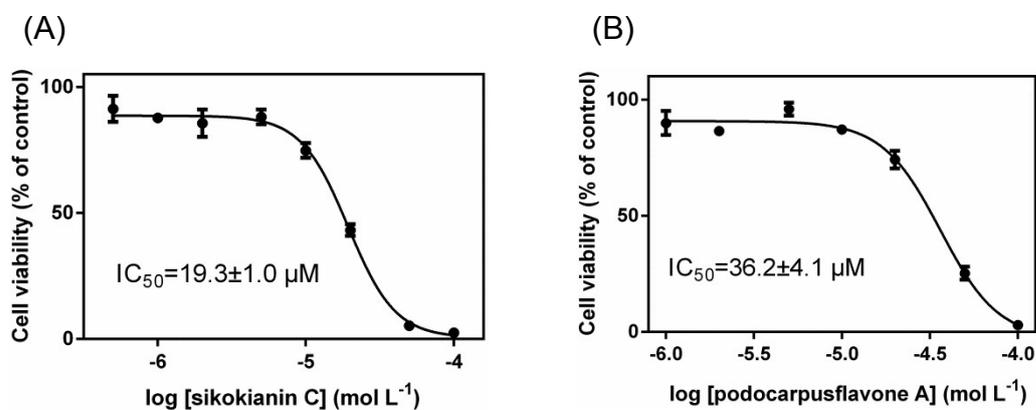
**Figure S7** H<sub>2</sub>S production was measured in NCM356 and HT29 cell lines by the methylene blue method. H<sub>2</sub>S production was stimulated in cell lysates by incubation at 37 °C (30 min) in presence of the CBS substrates cysteine (5 mM) and homocysteine (5 mM). CBS activity was significantly higher in HT29 cells, compared with NCM356 cells. AOAA (1 mM) blocked the H<sub>2</sub>S-producing activity of CBS in HT29 cells, whereas PAG (2 mM) had less effect on H<sub>2</sub>S production. The data were presented as the means ± SD (n=3).



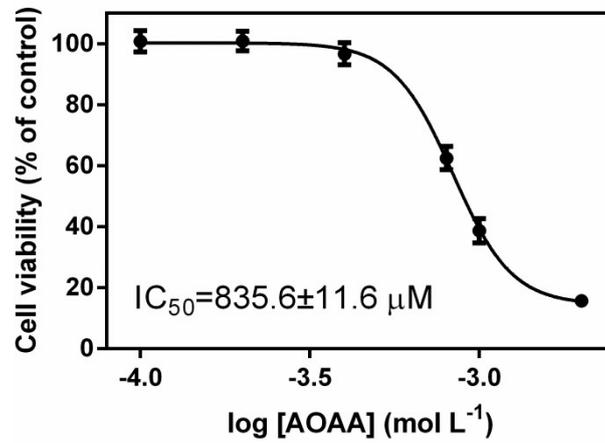
**Figure S8 Effect of AOAA on the viability of HT29 cancer cells.** The HT29 cancer cells that were cultured without AOAA were used as the control group, and the IC<sub>50</sub> value was determined according to the Methods. The data represented the cell viability of the experimental group compared with the control group and were presented as the means±SD (n=6). GraphPad Prism 6 software was used to fit the data.



**Figure S9** Effects of two selective hCBS inhibitors, 2'',4''-Di-O-(Z-p-coumaroyl) afzelin (A) and Agathisflavone (B), on the viability of HT29 colon cancer cells. The data represented the cell viability of the experimental group compared with the control group and were presented as the means±SD (n=6).

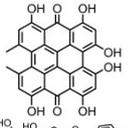
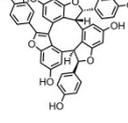
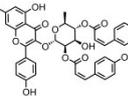
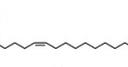
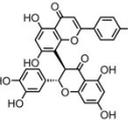
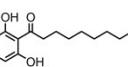
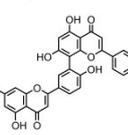
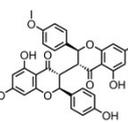
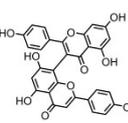
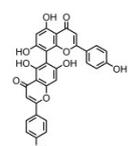
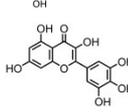
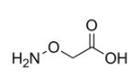


**Figure S10** Effects of two selective hCBS inhibitors, Sikokianin C (A), Podocarpusflavone A (B), on the viability of NCM356 cells. The data represented the cell viability of the experimental group compared with the control group and were presented as the means ± SD (n=6).



**Figure S11 Effect of AOAA on the viability of NCM356 cells.** The NCM356 cells that were cultured without AOAA were used as the control group, and the  $IC_{50}$  value was determined according to the Methods. The data represented the cell viability of the experimental group compared with the control group and were presented as the means $\pm$ SD (n=6). GraphPad Prism 6 software was used to fit the data.

**Table S1. Chemical structures and IC<sub>50</sub> values of the selected inhibitors**

Name	Compound structure	Molecular formula	IC <sub>50</sub> (μM) of hCBS	IC <sub>50</sub> (μM) of hCSE
Hypericin		C <sub>30</sub> H <sub>16</sub> O <sub>8</sub>	3.1±0.1	40.3±3.6
Caraphenol A		C <sub>42</sub> H <sub>28</sub> O <sub>9</sub>	5.9±0.3	12.1±1.3
2",4"-Di-O-(Z-p-coumaroyl) afzelin		C <sub>39</sub> H <sub>32</sub> O <sub>14</sub>	6.2±1.1	>400
NP-014428		C <sub>26</sub> H <sub>36</sub> O <sub>4</sub>	7.4±0.6	62±3.7
3'-Hydroxy-Volkensiflavon		C <sub>30</sub> H <sub>20</sub> O <sub>11</sub>	7.8±0.6	>400
NP-003872		C <sub>21</sub> H <sub>26</sub> O <sub>4</sub>	8.1±0.6	121.6±19.5
Podocarpusflavone A		C <sub>31</sub> H <sub>20</sub> O <sub>10</sub>	8.9±0.4	≥400
Sikokianin C		C <sub>31</sub> H <sub>24</sub> O <sub>10</sub>	9.2±1.6	>400
Cupressuflavone		C <sub>30</sub> H <sub>18</sub> O <sub>10</sub>	11.5±0.6	>400
Agathisflavone		C <sub>30</sub> H <sub>18</sub> O <sub>10</sub>	17.1±2.5	>400
Myricetin		C <sub>15</sub> H <sub>10</sub> O <sub>8</sub>	18.8±3.6	14.4±1.8
Aminooxyacetic acid (AOAA)		C <sub>2</sub> H <sub>5</sub> NO <sub>3</sub>	4.1±0.4 1.0±0.1*	3.7±0.6 0.57±0.03*

\*The IC<sub>50</sub> values were measured using 10 mM methylcysteine as the substrate in the presence of 15 μM CPM fluorescent probe.