Role of the cystathionine β-synthase/H_2S system in liver cancer cells and the inhibitory effect of quinolone-indolone conjugate QIC2 on the system

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Abstract. Hydrogen sulfide (H_2S), the third gasotransmitter, plays important roles in cancer biological processes. As endogenous H_2S exerts pro-cancer functions, inhibition of its production in cancer cells may provide a new cancer treatment strategy and be achieved via regulation of the function of cystathionine β-synthase (CBS), one of the main metabolic enzymes synthesizing H_2S. This enzyme plays important roles in the development and progression of colon and ovarian cancer, primarily regulating mitochondrial bioenergetics and accelerating cell cycle progression. In the present study, we firstly investigated the role of the CBS/H_2S system in human hepatoma cells, and then the inhibitory effect of a quinolone-indolone conjugate QIC2 on this system. When CBS was overexpressed in human hepatoma HepG2 and SMMC-7721 cells, inhibition of endogenous CBS/H_2S significantly reduced their viability and growth rate, as well as the proliferation of SMMC-7721 cells. Meanwhile, CBS knockdown caused multiple effects, including apoptosis of SMMC-7721 cells, an increase in the Bcl-2-associated X protein (Bax)/B cell lymphoma/leukemia (Bcl-2) ratio, activation of caspase-3 and polyADP-ribose polymerase (PARP), when compared with the scramble siRNA (Sc siRNA)-transfected groups. Heme oxygenase-1 (HO-1; a microsomal enzyme) expression was significantly decreased while the reactive oxygen species (ROS) level was increased in the CBS siRNA-transfected SMMC-7721 cells. QIC2 significantly reduced SMMC-7721 cell viability in a dose-dependent manner and showed a lower toxicity in human normal liver HL-7702 cells relative to the positive controls sunitinib and doxorubicin (DOX). The compound also inhibited cell proliferation and induced cell apoptosis in SMMC-7721 cells. Further analysis indicated that QIC2 downregulated the CBS/H_2S system, decreased both HO-1 protein and glutathione (GSH) levels while increased the ROS level and activated the caspase-3 cascade. Collectively, our results demonstrated that the CBS/H_2S system plays important roles in human hepatoma cells and QIC2 significantly inhibited cell growth via downregulation of the system.

Introduction

Chemotherapy plays a significant role in the treatment of liver cancer since the majority of patients diagnosed with liver cancer are terminal, and the tumors cannot be completely removed. At this stage, liver cancer has low susceptibility to chemotherapeutic drugs, an issue that may relate to multidrug resistance (MDR) genes. The commonly used clinical chemotherapeutic drugs for liver cancer therapy include cisplatin (PDD), doxorubicin (DOX), mitomycin (MCC), 5-fluorouracil (5-FU) and bleomycin (BLM). However, application of these drugs is limited to some extent due to their serious side effects. DOX is the most widely used drug in the treatment of liver cancer, but its cardiac toxicity is also the most severe among the chemotherapeutic drugs. Therefore, searching for new targets and screening for novel anti-liver cancer drugs is crucial.

Hydrogen sulfide (H_2S) is named as the third gasotransmitter signaling molecule alongside nitric oxide (NO) and carbon monoxide (CO), and plays important roles in many physiological processes (1-6). Recently studies on H_2S have revealed its roles in cancer therapy and have shown that H_2S exhibits both pro-apoptotic and anti-apoptotic effects in cancer cells (7-9). Moreover, tumor-derived endogenous H_2S contributes to angiogenesis and cytoprotection (10,11).

Endogenous H_2S is mainly generated by pyridoxal-5-phosphate (PLP)-dependent enzymes, cystathionine-β-synthase (CBS), cystathionine γ-lyase (CSE), and pyridoxal 5-phosphate (PLP)-independent enzyme, 3-mercaptopyruvate sulfurtransferase (MST) (12). Recent studies have demonstrated that endogenous H_2S produced by CBS promotes the proliferation of human ovarian and colon cancer cells (10,11). However,

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CBS primarily localizes in liver and brain tissues in humans and it is unclear whether it contributes to liver cancer progression. In the present study, we investigated the role of CBS in human liver cancer cells and the inhibitory effects of a novel inhibitor on CBS in these cells.

Materials and methods

Cell lines. Human liver cancer cell lines SMMC7721, HepG2, BEL-7404 and human normal liver cells HL7702 and QSG7701 were procured from the American Type Culture Collection (ATCC; Manassas, VA, USA), cultivated in Roswell Park Memorial Institute (RPMI)-1640 or Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a 37˚C incubator with 5% CO_2.

siRNA transfection. CBS-specific siRNA (sense, 5'-CCAAGU GUGAGUUCUUCAAdTdT-3' and antisense, 5'-UUGAAGA ACUCACACUUGGdTdT-3') was designed to target the open reading frame (ORF) region of the CBS mRNA. The cells were seeded to reach 30-40% confluency at the point of transfection. Forward transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Western blot analysis. Cells were harvested and lysed using RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM sodium chloride; 1.0% NP-40; 0.5% sodium deoxycholate; and 0.1% SDS) with 10 μg/ml protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were clarified by centrifugation at 12,000 x g for 10 min and protein concentration in the supernatant was determined. Total cellular proteins (40 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA). After blocking with 5% milk or 5% bovine serum albumin (BSA) for 2 h at room temperature, the membranes were incubated overnight at 4˚C with the primary antibodies. After incubation with the secondary antibody, the proteins were detected using AlphaImager chemiluminescence system (ProteinSimple, San Jose, CA, USA). The primary antibodies used included CBS rabbit polyclonal (1:1,000; Abcam, Cambridge, MA, USA), Bax rabbit monoclonal (1:1,000), Bcl-2 rabbit monoclonal (1:1,000), caspase-3 rabbit monoclonal (1:1,000), cleaved caspase-3 rabbit monoclonal (1:1,000), PARP rabbit monoclonal (1:1,000), cleaved PARP rabbit monoclonal antibodies (1:1,000) (all from Cell Signaling Technology, Danvers, MA, USA), HMOX1 (HO-1) rabbit polyclonal (1:600) and β-actin rabbit polyclonal antibodies (1:2,000) (both from Proteintech, Chicago, IL, USA). Secondary antibody was peroxidase-conjugated AffiniPure goat anti-rabbit/mouse (1:5,000; Proteintech).

H_2S detection. Production of H_2S from cancer cells was spectrophotometrically measured. Briefly, SMMC7721 cells were transfected with CBS siRNA or exposed to aminooxyacetate (AOAA; Sigma-Aldrich) or QICs (QIC0, QIC1, QIC2 and QIC3, quinolone-indolone conjugates (13), synthesized chemically by Professor Quoqiang Hu, College of Pharmacy, Henan University) and treated with 2 mM L-cysteine and 0.5 mM pyridoxal phosphate. Meanwhile 1% (w/v) zinc acetate (500 μl) was added to the filter papers adhered to the tissue culture plate cover to absorb H_2S. After 48 h, the filter papers were placed in tubes containing 0.2% (w/v) *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride dye (500 μl), 10% (w/v) ammonium ferric sulfate (50 μl) and 3 ml H_2O, and incubated for 20 min at room temperature. Absorbance at 670 nm was subsequently monitored. Production of H_2S was determined using a standard curve for NaHS (0-1 mM; R^2=0.9995) and presented as nmol/min/1x10^6 cells.

MTS and EdU assays. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and 5-ethynyl-2'-deoxyuridine (EdU) assays were used to assess cell viability and cell proliferation. SMMC7721 cells were plated into 96-well plates, and treated with CBS siRNA, CBS inhibitor aminooxyacetic acid (AOAA), CBS-overexpression plasmid and QICs (QIC0, QIC1, QIC2 and QIC3) for 48 h, respectively. Cell viability was evaluated by determining the number of cells with MTS (Sigma-Aldrich). The assay was carried out in triplicate for three independent experiments. DOX and sunitinib served as positive controls. The effects of CBS knockdown, CBS overexpression and QIC2 on cell proliferation were tested by the EdU assay kit (Ruibobio, Guangzhou, China). Briefly, cells were cultured in 96-well plates and transfected with CBS siRNA or the CBS-overexpression plasmid and exposed to AOAA or QICs for 48 h. Then, the cells were incubated with 50 μm of EdU for 2 h at 37˚C. Cells were fixed with 4% formaldehyde for 30 min, incubated with glycine (2 mg/ml) for 5 min and treated with 0.5% Triton X-100 for 10 min to permeabilize the cells. After being washed with phosphate-buffered saline (PBS) for 5 min, cells were incubated with Apollo for 30 min and treated twice with 0.5% Triton X-100. DNA was stained with Hoechst 33342 stain for 30 min and visualized with fluorescence microscopy. Five groups of cells in the images were randomly selected.

Flow cytometric assay. CBS siRNA- or scramble siRNA (Sc siRNA)-transfected cells, empty vector- or CBS-overexpressing plasmid-transfected cells and QIC2-treated or untreated cells (3x10^5 cells/well) were harvested at 48 h post-transfection or post-treatment. Apoptosis was determined via dual staining with Annexin V-FITC/PI (Biyuntian, Shanghai, China). Briefly, the cells were harvested and washed with PBS twice, and resuspended in 195 μl binding buffer. Annexin V-FITC (5 μl) was firstly added and gently mixed. Then, 10 μl PI was added, mixed gently and incubated for 10-20 min in the dark and analyzed using the FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA). The assays were carried out in triplicate.

Measurements of intracellular reactive oxygen species (ROS) and reduced glutathione (GSH). Production of intracellular ROS was quantified using the DCFH-DA assay according to the instructions included in the Reactive Oxygen Species Assay kit (Biyuntian). For the CBS siRNA- or Sc RNA-transfected cells, empty vector- or CBS-overexpression plasmid-transfected cells and QIC2-treated or untreated cells in a 96-well plate, old media were aspirated out and incubated in new RPMI-1640

medium containing 10 μM dichlorofluorescein diacetate (DCFH-DA) at 37˚C in a 5% CO_2 atmosphere for 20 min. Then, the medium was removed gently and replaced with 200 μl of 1X PBS followed by a wash with 1X PBS. The fluorescence released was read using a microplate spectrofluorometer with excitation/emission wavelengths set at 488/525 nm. Reduced GSH was determined using a GSH and GSSG assay kit (Biyuntian). The cells pretreated with QIC2 (0, 0.25, 0.5 and 1 μM) were collected and washed with 1X PBS. The pellet was immediately resuspended with three times volume of protein removal reagent M. Then, the sample was rapidly frozen and thawed twice using liquid nitrogen and 37˚C water bath. The suspension of cells was transferred into a microfuge tube and centrifuged at 10,000 g/min for 10 min at 4˚C. The clarified supernatant was collected for the assay according to the protocol. The assays were carried out in triplicate.

Statistical analysis. Statistical analyses were performed with the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The results are expressed as the mean ± SD. Differences between two groups were analyzed using the Student's t-test. $p<0.05$ was considered to indicate a statistically significant difference.

Results

Endogenous CBS/H_2S pathway modulates the proliferation of human hepatoma cells. To explore the relationship between CBS expression and the proliferation of hepatoma cells, we firstly checked the intrinsic levels of CBS protein in hepatoma cell lines including SMMC-7721, HepG2 and BEL-7404 as well as the normal liver cell lines HL-7702 and QSG-7701. As shown in Fig. 1A, CBS protein levels in the human hepatocellular carcinoma SMMC-7721 and HepG2 cells were higher than levels in the human normal liver HL-7702 and QSG-7701 cells. However, BEL-7404 cells had a low CBS protein level. CBS levels were reported to be coordinately regulated with proliferation in human cells and may represent a novel marker for both differentiation and proliferation in certain cell types (14). Previous research has also shown that the rise in H_2S released from hepatoma HepG2 cells is correlated with an increase in cystathionine β-synthase (CBS) (15). The high CBS expression in SMMC-7721 and HepG2 cells suggests that CBS is mainly responsible for the production of endogenous H_2S in the cells and is closely related to cell proliferation. The low CBS level in BEL-7404 cells may imply that other enzymes are mainly responsible for the catalysis of the generation of endogenous H_2S in these cells or that endogenous H_2S is not relevant to the survival and growth of BEL-7404 cells since previous studies have shown that CSE is also present in liver cancer cells (6). To explore the biological significance of high CBS expression in SMMC-7721 and HepG2 cells, we knocked down CBS using siRNA transfection or inhibited the activity of CBS protein by AOAA in SMMC-7721 cells and analyzed the changes in the endogenous H_2S level and cell proliferation ability. Surprisingly, we found that inhibition of endogenous CBS/H_2S significantly reduced the cell viability and inhibited the growth of SMMC-7721 cells (Fig. 1B-F). Moreover, EdU assay showed that CBS siRNA transfection also distinctly inhibited the proliferation of SMMC-7721 cells (Fig. 1G). These results indicate that the CBS/H_2S system is closely related to the cell survival and proliferation of human liver cancer cells and CBS downregulation is conducive to the treatment of liver cancer.

CBS knockdown induces cell apoptosis and increases oxidative stress in hepatoma cells. Apoptosis is one of the major mechanisms of inhibiting cell proliferation or inducing cell death. To explore how the CBS/H_2S pathway regulates the proliferation of hepatoma cells, we investigated the effect of CBS knockdown on cell apoptosis. As shown in the flow cytometric results (Fig. 2A), CBS siRNA transfection caused an increased apoptosis rate in the SMMC-7721 cells. We also examined changes in apoptosis-related proteins and found that CBS knockdown caused not only a distinct increase in the Bax/Bcl-2 ratio, but also activation of caspase-3 and PARP (Fig. 2B). Cancer cell survival is known to be related with the ability of cancer cells to counteract oxidative stress, which may be associated with Heme oxygenase-1 (HO-1), a microsomal enzyme that plays the role of an antioxidant to resist oxidative stress. HO-1 is overexpressed in hepatoma cells. It is also known that H_2S regulates resistance to oxidative stress in many organs and systems and plays an important regulatory role in the expression of HO-1 (16). However, whether or not the CBS/H_2S system maintains hepatoma survival by modulating HO-1 expression and oxidative stress remains unclear. To address this issue, we investigated the correlation between the CBS/H_2S system and HO-1 expression in hepatoma cells. Our data showed that HO-1 protein expression was significantly decreased in the CBS siRNA-transfected SMMC-7721 cells compared with the Sc siRNA-transfected group (Fig. 2C). Meanwhile, increased ROS level was also observed in the CBS siRNA-transfected SMMC-7721 cells (Fig. 2D). These results indicate that the CBS/H_2S system regulates cell apoptosis and is closely related to the oxidative stress in hepatoma cells.

QIC2 inhibits cell proliferation and promotes cell apoptosis by downregulating CBS expression in human hepatoma cells. AOAA, an inhibitor of CBS, is unable to be developed into a powerful anticancer drug, due to the cytotoxicity of AOAA in human normal liver HL-7702 cells and the high dose of AOAA needed in liver cancer cells (Fig. 3A). Thus, to screen more effective and less harmful CBS inhibitors for liver cancer therapy, we investigated the inhibitory effect of quinolone-indolone conjugates (QIC0, QIC1, QIC2 and QIC3) on the expression of CBS protein and cell viability in SMMC-7721 cells. As shown in Fig. 3B, the results clearly showed that a significant reduction in the CBS protein level was achieved under QIC2 treatment. However, treatments with QIC0, QIC1 and QIC3 did not exhibit obvious effects on CBS protein expression. In terms of cell growth, QIC2 yielded the strongest inhibitory effect on the SMMC-7721 cells compared with QIC0 ($p=0.018$), QIC1 ($p=0.008$) and QIC3 ($p=0.0009$) (Fig. 3C). QIC2 was thus used for further evaluation of the anticancer activity and relevant molecular mechanisms. Following treatment of QIC2, SMMC-7721 cell viability was significantly reduced in a dose-dependent manner. The inhibitory effect was stronger than that of the positive control sunitinib but slightly weaker than the positive control DOX (Fig. 3D). However, QIC2 demonstrated the lowest toxicity to human normal liver HL-7702 cells compared with both controls

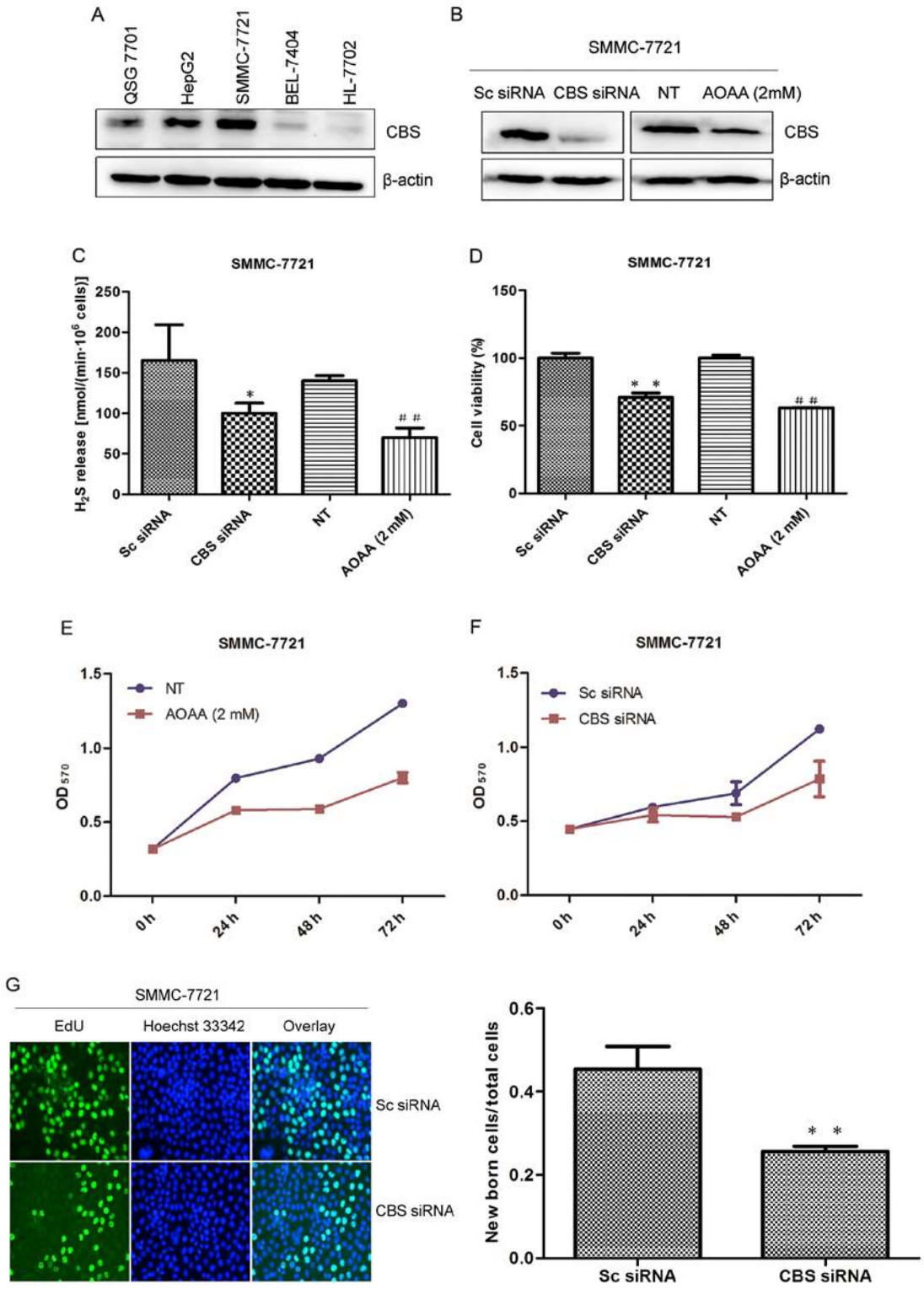

Figure 1. Expression of CBS and analyses of cell growth and proliferation associated with the inhibition of CBS/H_2S system in human hepatoma cells. (A) Expression level of CBS in human hepatoma and normal liver cells were detected by Western blotting (WB). (B) CBS protein level in SMMC-7721 cells transfected with CBS siRNA and treated with CBS inhibitor AOAA was determined by WB. (C) The effects of CBS siRNA and CBS inhibitor AOAA on the production of endogenous H_2S in human hepatoma SMMC-7721 cells were assessed spectrophotometrically using *N*,*N*-dimethyl--phenylene diamine-dihydrochloride. Error bars indicate SD (n=3); $^{*}p<0.05$ vs. the Sc siRNA group, $^{\#\#}p<0.01$ vs. the NT (no treatment) groups. (D-F) MTS assays were used to examine the effect of CBS siRNA and CBS inhibitor AOAA on cell growth in SMMC-7721 cells. Cell viability was evaluated in triplicate by microplate spectrofluorometer. Error bars indicate SD (n=3); $^{**}p<0.01$ vs. the Sc siRNA groups; $^{\#\#}p<0.01$ vs. the NT (no treatment) groups. (G) EdU assays were used to test the effect of CBS siRNA on cell proliferation in SMMC-7721 cells (magnification, x20). EdU, 5-ethynyl-2'-deoxyuridine. Error bars indicate SD (n=3); $^{**}p<0.01$ vs. the Sc siRNA groups.

sunitinib and DOX (Fig. 3E). Meanwhile, we also observed the inhibition of cell proliferation (Fig. 3F and G) and induction of cell apoptosis (Fig. 3H and I). To demonstrate whether QIC2 acts through CBS, we transfected the CBS-overexpressing

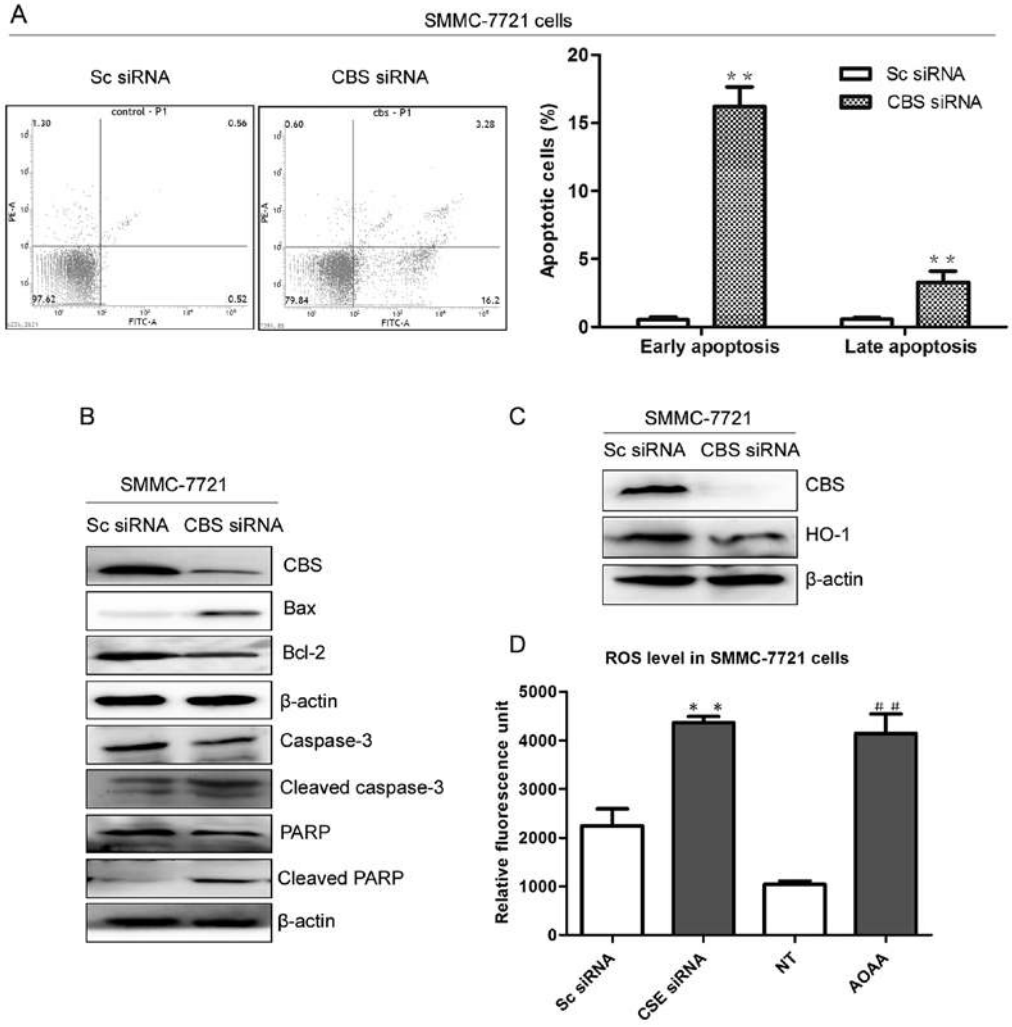

Figure 2. Analyses of cell apoptosis and oxidative stress associated with CBS downregulation in SMMC-7721 cells. (A) Flow cytometric assay with Annexin V/FITC double staining. Error bars indicate SD (n=3); $^{**}p<0.01$ vs. the Sc siRNA groups. (B) The effect of CBS downregulation on apoptotic-related protein expression by western blot analysis (WB). (C) Analyses of oxidative stress associated with CBS downregulation. HO-1 expression was detected by WB. (D) ROS levels were evaluated. Error bars indicate SD (n=3); $^{**}p<0.01$ vs. the Sc siRNA groups, $^{\#\#}p<0.01$ vs. the NT (no treatment) groups.

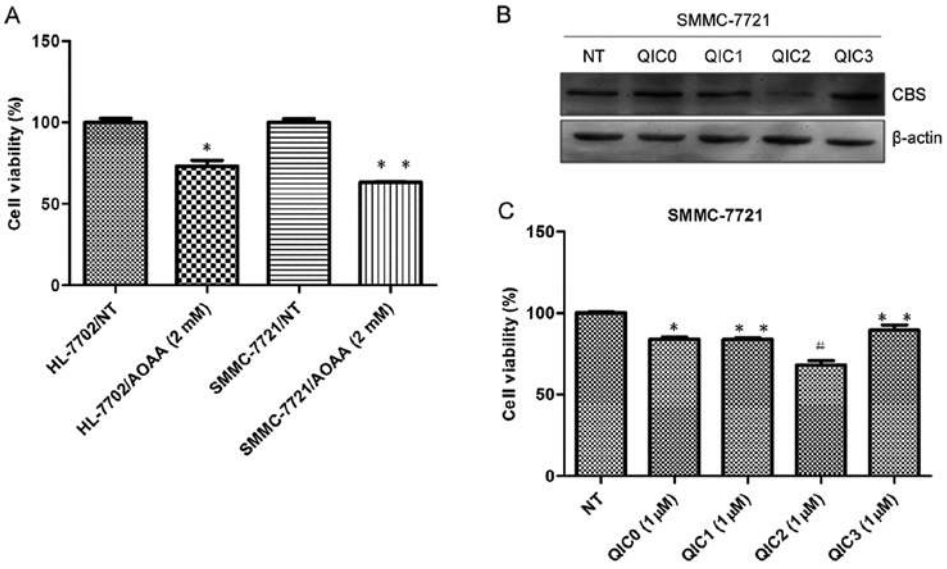

Figure 3. The effect of QIC2 with CBS inhibitory activity on cell proliferation and apoptosis in SMMC-7721 cells. (A) AOAA caused growth inhibition in human normal liver HL-7702 cells and hepatoma SMMC-7721 cells. Cell viability was evaluated in triplicate by a microplate spectrofluorometer. Error bars indicate SD (n=3); $^{*}p<0.05$ vs. the HL-7702/NT (no treatment) groups, $^{**}p<0.01$ vs. the SMMC-7721/NT (no treatment) groups. (B and C) Screening of effective and safe CBS inhibitors from quinolone-indolone conjugates (QICs). Error bars indicate SD (n=3); $^{\#}p<0.01$ vs. the NT (no treatment) groups, $^{*}p<0.05$ vs. the QIC2 groups, $^{**}p<0.01$ vs. the QIC2 groups.

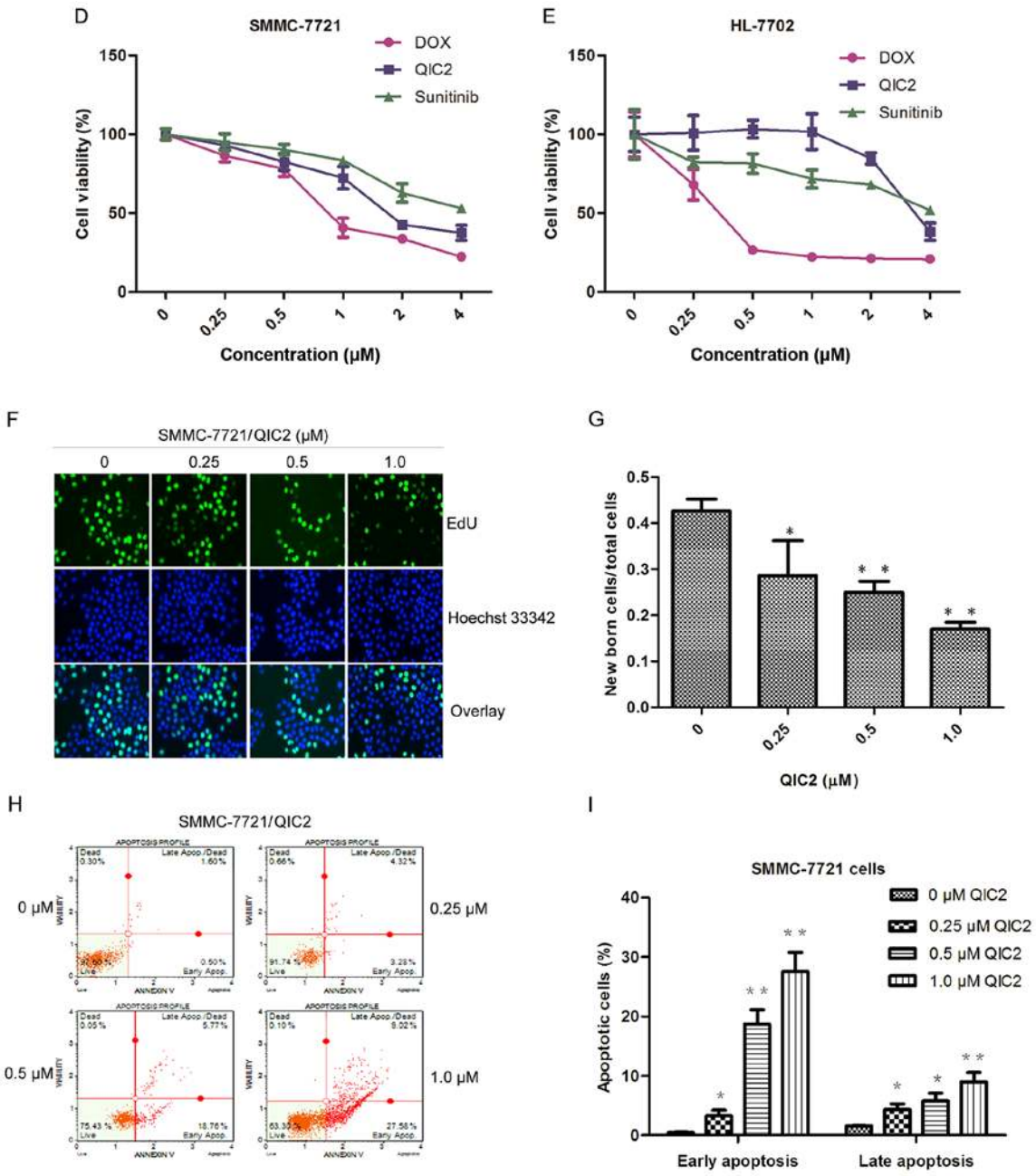

Figure 3. Continued. (D and E) Effects of QIC2 on cell growth in human liver cancer SMMC-7721 and normal liver HL-7702 cells. (F and G) Analyses of cell proliferation. EdU assays were used to test the effect of QIC2 on proliferation in SMMC-7721 cells (magnification, x20). EdU, 5-ethynyl-2'-deoxyuridine. Error bars indicate SD (n=3); *p<0.05 vs. the 0 μM QIC2 groups, **p<0.01 vs. the 0 μM QIC2 groups. (H and I) Analyses of cell apoptosis. Flow cytometric assay with Annexin V/7-amino-actinomycin D double staining. Error bars indicate SD (n=3); *p<0.05 vs. the 0 μM QIC2 groups, **p<0.01 vs. the 0 μM QIC2 groups. AOAA, aminooxyacetate; DOX, doxorubicin; QIC2, quinolone-indolone conjugate 2.

plasmid into SMMC-7721 cells and determined the activities and roles of QIC2 in the cells. Increased cell proliferation and decreased ROS level were observed in the cells transfected with the CBS plasmid compared with the cells transfected with the empty vector (Fig. 4), which further manifests the roles of CBS in hepatoma cells. Meanwhile, the decreased cell viability and newborn cells caused by QIC2 were not distinct between the CBS plasmid- and empty vector-transfected groups (Fig. 4A-C), suggesting that CBS overexpression did not abrogate the inhibitory activity of QIC2 on cell growth and proliferation. However, the increase in the percentage of apoptotic cells and elevated ROS levels induced by QIC2 were significantly reduced in the CBS-transfected group when compared with the vector-transfected group (Fig. 4D-F). This infers that CBS overexpression rescues the effects of QIC2 on cell apoptosis and QIC2 acts by targeting CBS.

QIC2 inhibits the CBS/H_2S system, causes oxidative stress and activates the caspase-3 cascade in human hepatoma cells. To further investigate the anticancer mechanism of QIC2, we evaluated changes in the CBS/H_2S system, HO-1 protein expression, ROS and GSH levels in the SMMC-7721 cells. Downregulated CBS/H_2S system, decreased HO-1 protein level while increased ROS level were found in the

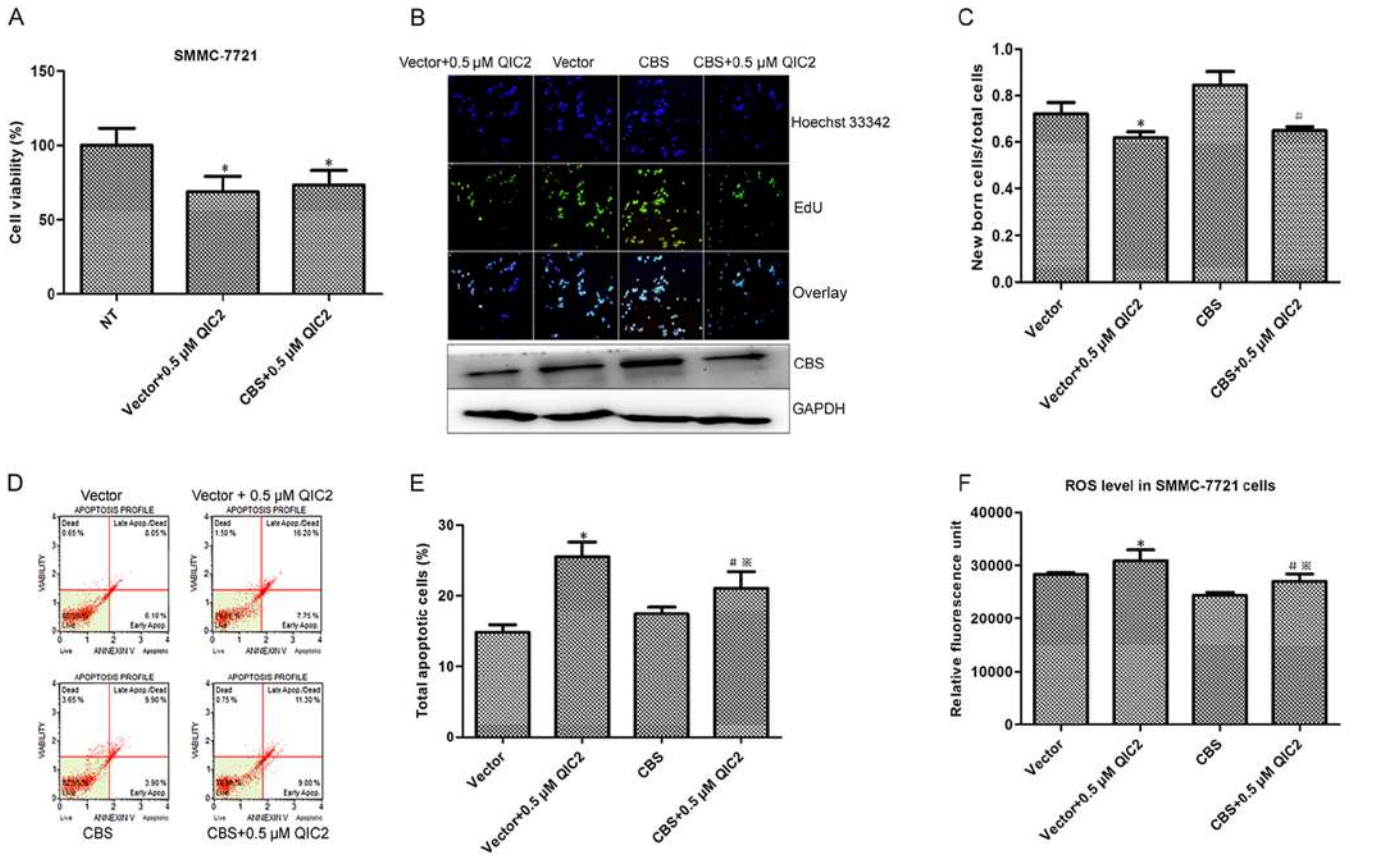

Figure 4. Effects of CBS overexpression on the roles of QIC2 in SMMC-7721 cells. (A) Cell viability analysis. MTS was performed in triplicate by a microplate spectrofluorometer. Error bars indicate SD (n=3); *p<0.05 vs. the NT (no treatment) groups. (B) Analyses of cell proliferation and CBS expression. EdU assay and western blot analysis (WB) were performed (magnification, x20). (C) Statistical analysis of EdU results. Error bars indicate SD (n=3); *p<0.05 vs. the vector groups, $^{\#}$p<0.05 vs. the CBS groups. (D and E) Analyses of cell apoptosis. Flow cytometric assay with Annexin V/7-amino-actinomycin D double staining. Error bars indicate SD (n=3); *p<0.05 vs. the vector groups, $^{\#}$p<0.05 vs. the CBS groups, $^{※}$p<0.05 vs. the vector + 0.5 μM QIC2 groups. (F) ROS levels were evaluated. Error bars indicate SD (n=3); *p<0.05 vs. the vector groups, $^{\#}$p<0.05 vs. the CBS groups, $^{※}$p<0.05 vs. the vector + 0.5 μM QIC2 groups. EdU, 5-ethynyl-2'-deoxyuridine. CBS, CBS-overexpression plasmid.

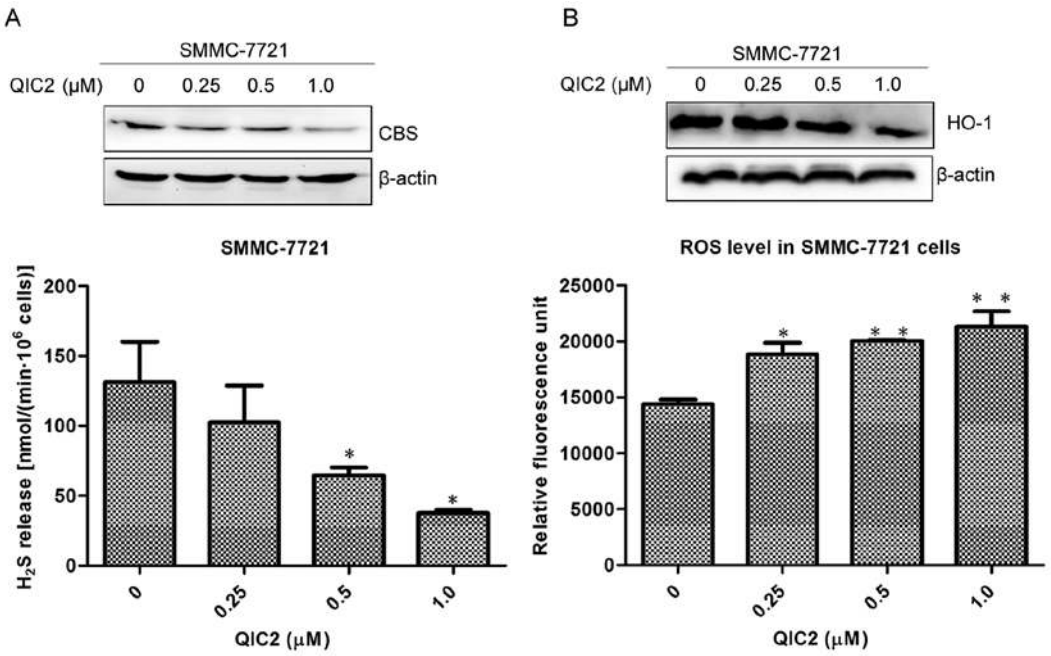

Figure 5. Effects of QIC2 on the CBS/H_2S system and oxidative stress in SMMC-7721 cells. (A) Inhibition of different concentrations of QIC2 on the CBS/H_2S system. Error bars indicate SD (n=3); *p<0.05 vs. the 0 μM QIC2 groups. (B) The effect of different concentrations of QIC2 on HO-1 expression and ROS levels. Western blot analysis (WB) was used to detect HO-1 expression. The production of intracellular ROS was quantified using the DCFH-DA assay. Error bars indicate SD (n=3); *p<0.05 vs. the 0 μM QIC2 groups, **p<0.01 vs. the 0 μM QIC2 groups.

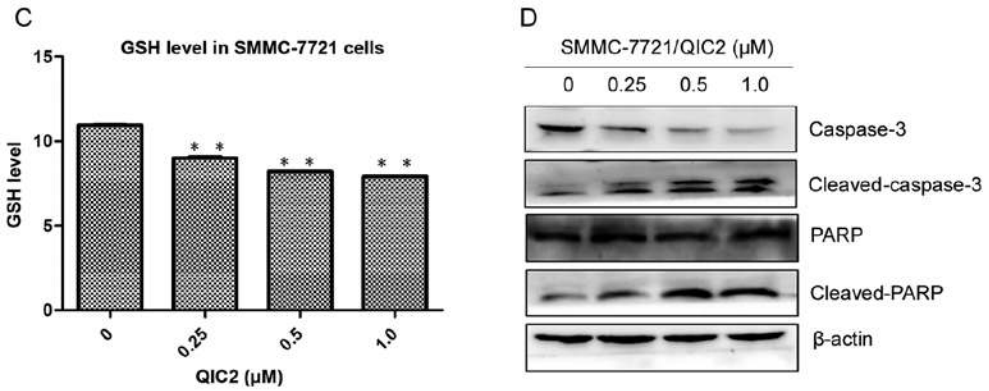

Figure 5. Continued. (C) The effect of different concentrations of QIC2 on the GSH level. Reduced GSH was determined by GSH and GSSG assay kit. Error bars indicate SD (n=3); $^{**}p<0.01$ vs. the 0 μM QIC2 groups. (D) The effect of different concentrations of QIC2 on the caspase-3 cascade. Western blot analysis (WB) was used to detect expression of caspase-3, cleaved caspase-3, PARP and cleaved PARP.

SMMC-7721 cells treated with QIC2 (Fig. 5A and B). This was accompanied by a decreased GSH level (Fig. 5C), suggesting that QIC2 causes oxidative stress in SMMC-7721 cells. Additionally, QIC2 was found to activate caspase-3 and PARP in the SMMC-7721 cells (Fig. 5D). These results indicate that QIC2 induces human hepatoma cell apoptosis via increasing oxidative stress and activating the caspase-3 cascade due to inhibition of the CBS/H_2S system.

Discussion

The CBS/H_2S system is strongly expressed in various ovarian and colon cancer cell lines and contributes to cancer progression. In the present study, we demonstrated that CBS protein was also highly expressed in human hepatoma cell lines HepG2 and SMMC-7721. This phenomenon led us to believe that CBS/H_2S is involved in the proliferation of HCC cells. Indeed, inhibition of the CBS/H_2S pathway by AOAA or CBS siRNA strongly suppressed the proliferation of the HepG2 and SMMC-7721 cells. This additionally suggests that the CBS/H_2S pathway contributes to the excessive growth of hepatoma cells with high intrinsic CBS expression levels.

Apoptosis is one of the major mechanisms involved in the inhibition of cell proliferation or induction of cell death (17-21), and involves proteomic variations, including the Bcl-2 family, Bax and the caspase-3 cascade. In our experiments, inhibition of the CBS/H_2S system caused by CBS siRNA induced the apoptosis of SMMC-7721 cells, pointing to the involvement of the CBS/H_2S pathway in modulating hepatoma cell proliferation. In addition, we found that CBS knockdown caused a distinct increase in the Bax/Bcl-2 ratio. While in CBS-knockdown cells, caspase-3 and PARP activities were significantly upregulated. All these observations were consistent with the molecular indices of cell apoptosis. Therefore, we conclude that inhibition of the CBS/H_2S pathway causes apoptosis via increasing the ratio of Bax/Bcl-2 and activating the caspase-3 cascade.

Linking the CBS/H_2S system with HO-1 expression and oxidative stress in cancer cells has not been illustrated previously. Cancer cell apoptosis involves oxidative stress while HO-1 plays vital antioxidant and anti-apoptosis roles. Therefore, we assume that the CBS/H_2S system is involved in HO-1 expression and oxidative stress in hepatoma cells. Indeed, we found that the downregulation of the CBS/H_2S system inhibited the expression of HO-1 and increased the oxidative stress level in SMMC-7721 cells. Previous studies have shown that ROS display a 'two-faced' character in cancer cells (22-24). On the one hand, a basal level of ROS induces and sustains the oncogenic phenotype of cancer cells. On the other hand, an increased ROS level inhibits cancer progression. Oxidative stress induced by increased ROS is a proverbial inducer of apoptosis in human cancer cell lines (25-28). In human hepatoma cells, we observed that inhibition of CBS/H_2S increased ROS production, a result similar to the inhibition of CSE/H_2S (29). These findings suggest that the CBS/H_2S system regulates oxidative stress and consequently induces the apoptosis of hepatoma cells.

Thus, we demonstrated that the CBS/H_2S system with high intrinsic CBS expression levels is vital in maintaining the proliferation of hepatoma cells. It is expected that inhibition of the system restrains the excessive growth of hepatoma cells and induces the process of mitochondrial apoptosis. Based on CBS inhibitor screening assay, we identified a quinolone-indolone conjugate compound QIC2 with a strong suppressive effect on both CBS protein expression and hepatoma cell proliferation. In terms of the mechanism involved in the modulation of proliferation, QIC2 was found to induce cell apoptosis by increasing oxidative stress and activating the caspase-3 cascade via inhibition of the CBS/H_2S system.

In conclusion, the CBS/H_2S pathway is vital for maintaining the proliferation of hepatoma cells. The novel compound QIC2 can potently suppress the CBS/H_2S system to inhibit the excessive growth and proliferation of the cells. Further investigation of the roles of CBS in liver tumors and its relationship with pathological features of liver cancer may advance the development of CBS and its inhibitors into effective therapeutic approaches against liver cancer.

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