

Thioridazine elicits potent antitumor effects in colorectal cancer stem cells

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Abstract. As a member of the phenothiazine family, thioridazine (THIO) is a potent anti-anxiety and anti-psychotic drug. Recent studies have reported that THIO could suppress the growth of several types of cancer cells. However, the effect of THIO on colorectal cancer stem cells (CSCs) has not been investigated. In the present study, we examined the effect of THIO on viability of CSCs isolated from the human colon cancer cell line HCT116 and its colony-formation ability, along with its stem cell-specific gene expression. The CSCs, EpCAM⁺ and CD44⁺ subpopulations from HCT116 cells were isolated using immunomagnetic beads. After incubation with several concentrations of THIO, we evaluated the proliferation and invasion ability of colon CSCs, as well as cell apoptosis. We found that THIO significantly suppressed the proliferation and invasion of colon CSCs and induced cell apoptosis in a concentration-dependent manner. The expression of some apoptosis genes (Bax and caspase-3) was upregulated after treatment with THIO, while that of the anti-apoptosis gene Bcl-2 was downregulated. Moreover, the CSC mitochondrial membrane potential was downregulated. Overall, this study showed that THIO inhibits the proliferation of CSCs derived from the HCT116 cell line through induction of apoptosis, and thus, could be a promising agent for the treatment of colon cancer and worthy of exploring in prospective clinical studies.

Introduction

Thioridazine (10-[2-(1-methyl-2-piperidyl)ethyl]-2-methylthiophenothiazine, THIO), a member of the phenothiazine family, was originally used to treat psychotic disorders such as psychosis and schizophrenia (1-3). In addition, in advanced cancer patients, this drug has been used to treat cancer-related sweating (4,5), as well as depression (6). In recent years, however, some studies have demonstrated that THIO could

inhibit the growth of some cancer cell lines (7-14). It has also shown selectivity for cancer stem cells (CSCs), such as leukemic cancer stem cells (15), glioblastoma stem cells (3) and breast cancer stem cells (16). Thus, THIO is currently regarded as a drug with potential usefulness in anticancer chemotherapy (9,17).

Recent evidence suggests that cancer development is mainly driven by a rare population of cells, the CSCs (16,17). Moreover, some scientists argue that conventional chemotherapeutics are ineffective against human CSCs (18). Therefore, the development of new drugs targeting CSCs holds special significance in clinical cancer research. Sachlos *et al* (15) demonstrated that THIO could selectively target neoplastic cells and impair human somatic CSCs capable of *in vivo* leukemic disease initiation without any effect on normal blood stem cells. Their study identified the potential of THIO to target CSCs directly. However, its exact effect on CSCs of other types of cancers still requires further investigation.

Colon cancer is one of the most prevalent cancers, with incidence rates that have been increasing steadily worldwide (19,20). In recent years, despite a series of remarkable advances in colon cancer chemotherapy, the increased resistance to anticancer drugs has been a serious obstacle to the efficient treatment of the disease. Overcoming drug resistance and targeting CSCs are key for the improvement of chemotherapy response (15,20,21). Therefore, the development of novel effective drugs for colon cancer is urgently required.

In this study, we mainly investigated the anti-proliferation and anti-invasion effects of THIO on CSCs isolated from a human colon cancer cell line (HCT116) and further determined the underlying mechanisms. These findings may contribute to the development of THIO-based chemotherapy for patients with colon cancer resistant to traditional anticancer drugs.

Materials and methods

Cell lines and culture. The human colon cancer cell line HCT116 and the human lung fibroblast cell line HELF were obtained from the Cell Bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). HCT116 and HELF cells were maintained in RPMI-1640 medium (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin-streptomycin mixed solution. The CSCs isolated from HCT116 cells were cultured in DMEM/F12 medium

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(Gibco) with 10 $\mu\text{g/ml}$ EGF (Sigma-Aldrich, St. Louis, MO, USA), 10 $\mu\text{g/ml}$ bFGF (Sigma-Aldrich) and 1% B27 supplement (Gibco). The media were changed every other day. The cells were incubated in a humidified incubator with 5% CO_2 at 37°C, and were passaged by a dilution of 1:3 every 4 or 5 days.

EpCAM⁺ and CD44⁺ cell selection. The cell selection method followed the protocol published by Zhang *et al* (21). Briefly, HCT116 cells were rinsed with phosphate-buffered saline (PBS) and detached with trypsin at 37°C. After centrifugation, cells were first incubated with anti-human CD44 monoclonal antibody conjugated with biotin (eBioscience, Inc., San Diego, CA, USA), and then fractionated using a CELlection Biotin Binder kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. The isolated cells were further incubated with anti-human EpCAM monoclonal antibody conjugated with biotin (eBioscience) and positive cells were then isolated with the same kit. Cells were cultured in CSC medium, and the purity of such CSCs was evaluated by flow cytometry and immunohistochemistry.

Flow cytometric analysis. The CSCs were dissociated into single cells, further fixed with fixation buffer (eBioscience) and prepared at a concentration of $2.0 \times 10^6/\text{ml}$ of PBS. Anti-human CD44 antibody conjugated with FITC (BD Biosciences, San Jose, CA, USA) and anti-human EpCAM antibody conjugated with PE (BD Biosciences) were added and incubated for 30 min at 4°C. After washing twice with PBS, the cells were acquired and analyzed by FACScalibur (BD Biosciences).

Immunohistochemistry. The single CSCs were seeded into plates covered in Matrigel and cultured for 24 h. They were fixed in 4% formaldehyde for 20 min at 4°C and permeabilized with 0.1% Triton X-100 for 10 min at 25°C. For immunohistochemistry, the primary antibodies used were anti-human CD44 monoclonal antibodies (1:50; eBioscience) and anti-human EpCAM monoclonal antibodies (1:50; eBioscience). After 12-14 h of incubation at 4°C, samples were washed three times with PBS and processed using an ABC kit and DAB solution (both purchased from Vector Laboratories, Inc., Burlingame, CA, USA). Finally, the sections were imaged with an Axio Scope A1 and AxioCAM MRc 5 (Carl-Zeiss, Oberkochen, Germany).

Colony formation assay. The CSCs were dissociated into single cells and seeded into a 96-well plate at a concentration of 0.5 cells/well. Briefly, the cell suspension was diluted at concentration of 50 cells/ml and 100 μl of cell suspension was added into each well. As a result, there is 1 cell or 0 cell seeded into each well. We observed and imaged cell cloning. The plate was incubated in a humidified incubator with 5% CO_2 at 37°C and half of the medium was changed every 3 days. Colonies gradually formed over the next 3 weeks.

Inhibitory effects of THIO on cell proliferation. In order to analyze the anticancer effect of THIO on CSCs derived from HCT116 cells, a CCK-8 assay was performed. In order to explore the effect of THIO on fibroblast cells, representing one of the largest amount of cells in humans, HELF cells were also selected in CCK8-assay. The cells were seeded in 96-well

Table I. Real-time qPCR primer sequences.

Gene	Primer sequence (5'-3')
Nanog	F: ATGCCTGTGATTTGTGGGCC R: GCCAGTTGTTTTTCTGCCAC
CLDN-6	F: GCCAGATGCAGTGCAAGGTGT R: GATGACAAAGACAATCCCAGAGGTG
Bax	F: TAACCAAGGTGCCGGAAGTGA R: GGGAGGAGTCTCACCAACCA
Caspase-3	F: CATGGAAGCGAATCAATGGACT R: CTGTACCAGACCGAGATGTCA
Bcl-2	F: GGGGAGGATTGTGGCCTTCTTT R: TAATGTGCAGGTGCCGTTTCAG
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA

F, forward; R, reverse.

plates at a concentration of 1.0×10^5 cells/well, and were treated with THIO (Sigma-Aldrich) at different concentrations (0, 10, 20 or 50 μM) for 24 h. The medium without any cells was used as the blank group, while 100 μM cisplatin (DDP) was used as the positive control group. The proliferation index of each group was determined using the Cell Counting kit-8 (CCK-8; Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions (21). In brief, 10 μl of CCK-8 solution was added into each well (containing 100 μl of medium) and cultured for 1-2 h at 37°C. The absorbance at 450 nm, which was directly proportional to the number of living cells, was observed for each group ($n=4$). The inhibition ratio was used to measure cell proliferation in the present study and it was described as (absorbance of 0 μM group - absorbance of each experimental group)/(absorbance of 0 μM group - the absorbance of the blank group).

Cell invasion assay. To assess cell invasion, a Transwell system was used (pore size: 8 μm ; Corning, Inc., Corning, NY, USA) following the manufacturer's protocol. The CSCs were seeded onto the upper insert covered with Matrigel at a concentration of 1×10^5 cells per insert in serum-free medium. Outer wells were filled with RPMI-1640 medium containing 10% FBS as a chemoattractant. Then, cells were incubated for 48 h at 37°C. Non-invading cells were removed by swabbing the top layer, and cells able to migrate through the gel and attach to the lower surface of the membrane were stained with crystal violet. The number of cells in four randomly selected microscopy fields was counted for each filter.

Real-time qPCR. Total RNA was extracted using TRIzol reagent (Invitrogen). For each sample, RNA (2 mg) was reverse-transcribed using an RT-PCR kit (Takara, Shiga, Japan), and qPCR was performed with a Thermal Cycler Dice™ Real-Time System and SYBR-Green Premix EX Taq™ (Takara). In the present study, GAPDH was used for qPCR normalization, and all measurements were performed in triplicate. The primer sequences used (5'→3') are shown in Table I.

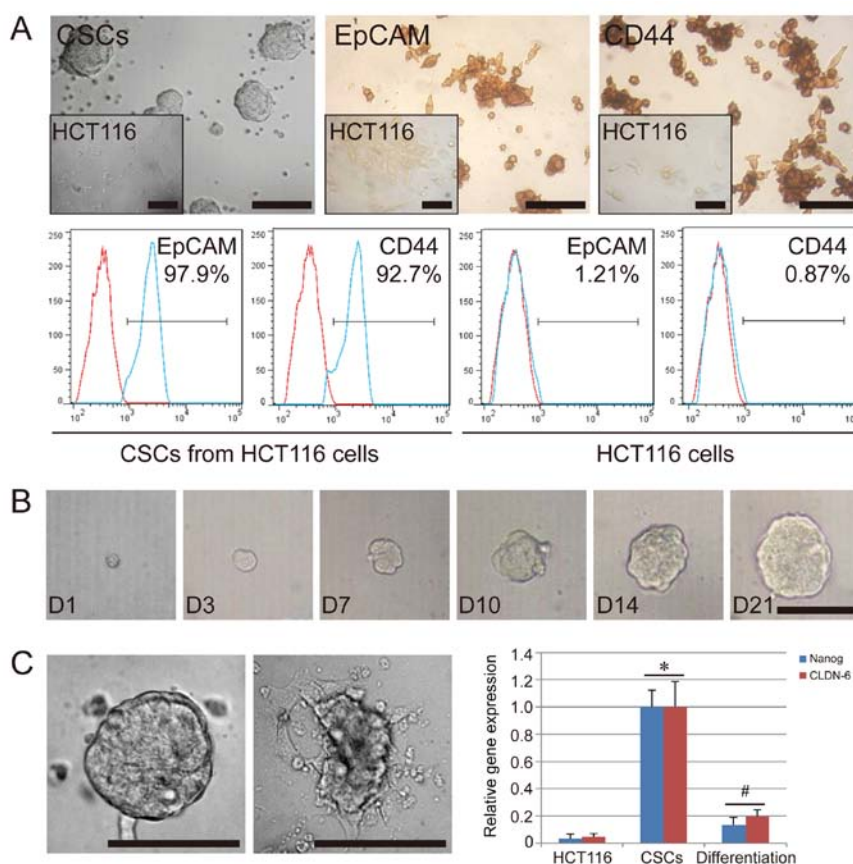


Figure 1. Characterization of CSCs isolated from the HCT116 cell line. (A) Appearance and immune phenotype of isolated CSCs. The CSCs isolated from HCT116 cells formed tumor spheres in suspension when cultured *in vitro*, and the cells were positive for EpCAM and CD44 under immunohistochemistry and FACS detection. HCT116 cells were used as the negative control group and were nearly negative for EpCAM and CD44. (B) Process of colony formation from one CSC. The CSC proliferated and grew gradually, and formed colonies after 21 days. (C) The necessity of a serum-free culture condition for maintaining stem cell characteristics of CSCs. Cultures under FBS conditions could lead to a layer of adherent confluent cells, as well as the downregulated expression of stem cell-specific genes (NANOG and CLDN-6) in CSCs. Results of qPCR are expressed as mean \pm SEM. A t-test was performed to compare the various groups and $P < 0.05$ was considered statistically significant. * $P < 0.05$ compared with the normal HCT116 group, and # $P < 0.05$ compared with the CSC group without any FBS treatment. Scale bars, 500 μ m.

Western blotting. Western blotting was carried out to test for cleaved caspase-3 using the same protocol as the one we reported in a previous publication (21). Briefly, cells were lysed with lysis buffer (50 mM Tris pH 7.0, 1 mM EDTA, 150 mM NaCl, 1% NP40, 10 mM NaF, 1 mM Na_3VO_4) containing protease inhibitor cocktail (Roche, Basel, Switzerland), and protein concentrations were determined using a BCA assay kit (Beyotime Institute of Biotechnology, Nanjing, China). Protein bands were separated by electrophoresis in a 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking them with 4% non-fat dry milk in Tris-buffered saline (TBS), the membranes were incubated overnight at 4°C with primary antibodies (rabbit anti-caspase-3; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1,000 in TBS. They were washed three times with TBS containing 0.5% Tween-20, and then incubated for 1 h at 25°C with secondary antibodies conjugated with a 1:5,000 dilution of horseradish peroxidase (HRP) in TBS. Membranes were then washed three times in TBS containing 0.5% Tween-20 at 25°C. Finally, protein bands were visualized on X-ray film using enhanced chemiluminescence (ECL; GE Healthcare, Bethesda, MD, USA).

Analysis of cell apoptosis. To analyze cell apoptosis, acridine orange/ethidium bromide (AO/EB) staining and Annexin V-FITC/PI staining were used according to the manufacturer's instructions (BD Biosciences). Moreover, to determine the effect of THIO on mitochondrial membrane potential in CSCs, JC-1 apoptosis detection kit (BD Biosciences) was used according to the manufacturer's instructions and assessed by fluorescence-activated cell sorting (FACS).

Statistical analysis. Statistical analysis was performed with the SPSS 17.0. The results are expressed as mean \pm SEM. The differences between the groups were assessed by one-way ANOVA followed by t-tests. $P < 0.05$ was considered statistically significant.

Results

Characterization of CSCs from HCT116 cells. The CSCs isolated from HCT116 formed tumor spheres in suspension when cultured *in vitro*. Their appearance was distinct from the original HCT116 cells, which were spindle-shaped and grew in adherent state (Fig. 1A). After three to four passages, the cells were further characterized by immunohistochemistry

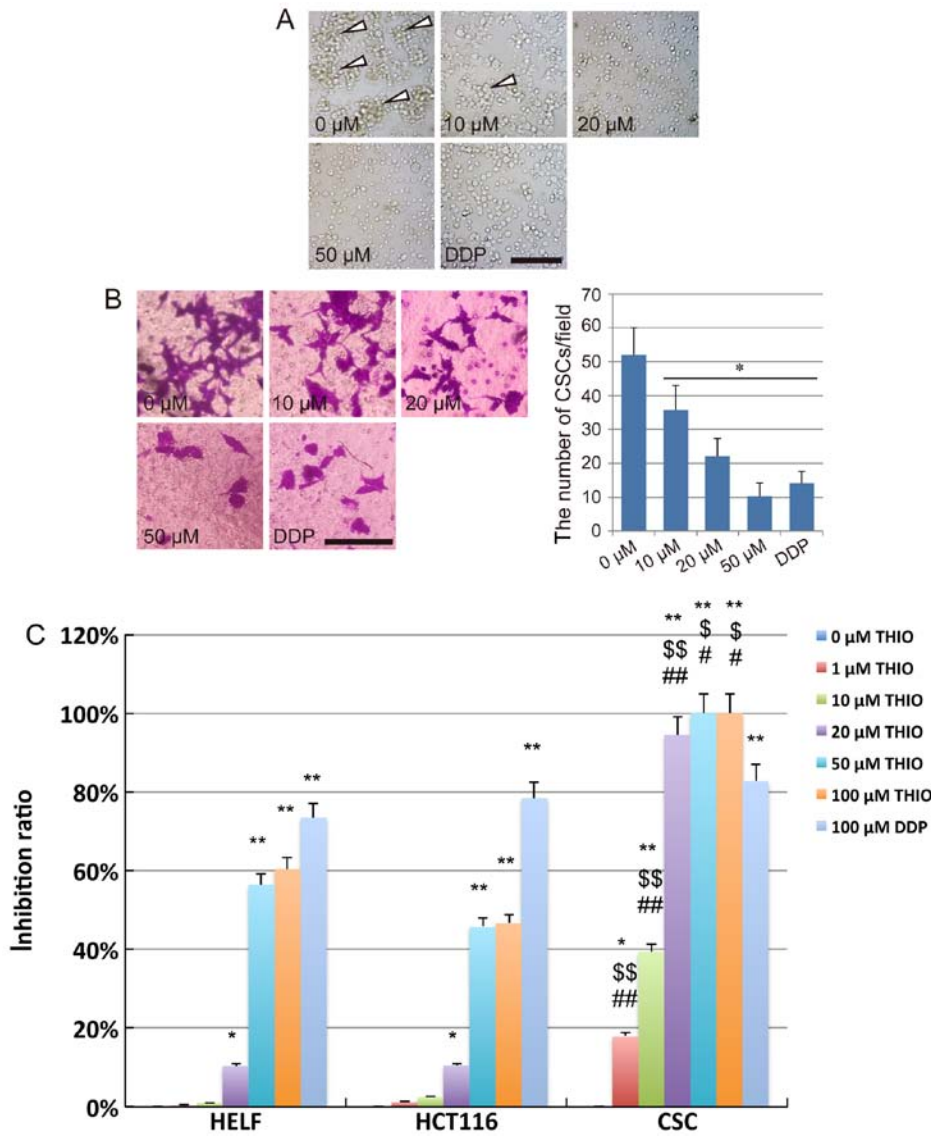


Figure 2. Effect of THIO on the proliferation and invasion of CSCs from HCT116 cells. (A) Effect of THIO on the proliferation ability of CSCs. The morphological alterations of each group are shown, and colony formation is marked by a white arrowhead. (B) Effect of THIO on the invasion ability of CSCs. DDP at a concentration of 100 μ M was used as the positive drug group. (C) Effect of THIO on the proliferation ability of HELF cells, HCT116 cells and CSCs from HCT116 cells. DDP at a concentration of 100 μ M was used as the positive drug group. Results are expressed as mean \pm SEM. A t-test was performed to compare the various groups, and $P < 0.05$ was considered statistically significant. * $P < 0.05$ compared with the THIO group at a concentration of 0 μ M, $^{\$}P < 0.05$, and $^{\$\$}P < 0.01$ compared with the THIO group at the same concentration in HELF cells. # $P < 0.05$ and ## $P < 0.01$ compared with the group of THIO at the same concentration in HCT116 cells. Scale bars, 500 μ m.

and flow cytometry. The results indicated that the CSCs used in the present study were positive for EpCAM (97.9%) and CD44 (92.7%), while HCT116 cells used as the negative control group displayed a low expression of EpCAM (1.21%) and CD44 (0.87%) (Fig. 1A). To further confirm the colony-forming ability of the CSCs, we separated the CSC spheres into single cells and analyzed their colony-formation ability in 96-well plates. As expected, a single CSC could proliferate and grow; in fact, over 40% of the CSCs formed a tumor sphere after 21 days of single-cell culture (Fig. 1B). In addition, we confirmed that the serum-free condition was necessary for the colony-formation ability and the stem cell-specific gene expression of CSCs. We compared the *in vitro* cell culture in basal conditions plus 10% FBS and in serum-free conditions. Cultures under FBS conditions could lead to a layer of adherent confluent cells. Compared with HCT116 cells, the

CSCs showed a high expression of stem cell-specific genes, NANOG, and CLDN-6 ($P < 0.05$), while downregulation of these genes occurred in the CSCs cultured under FBS conditions ($P < 0.05$) (Fig. 1C). These results suggest that the tumor sphere-like colonies could be obtained from the HCT116 cell line, and that these cells had some stem cell characteristics. In serum-free medium supplemented with EGF and bFGF, the CSCs differentiated even under conditions of an extra-low cell concentration, such as single cell conditions, ruling out the possibility that CSCs may aggregate owing to the high concentration of cells in cultures.

Effect of THIO on the proliferation and invasion of CSCs from HCT116 cells. After treatment with THIO for 24 h at different concentrations, severe morphological alterations were observed in the majority of CSCs cells. We found that the

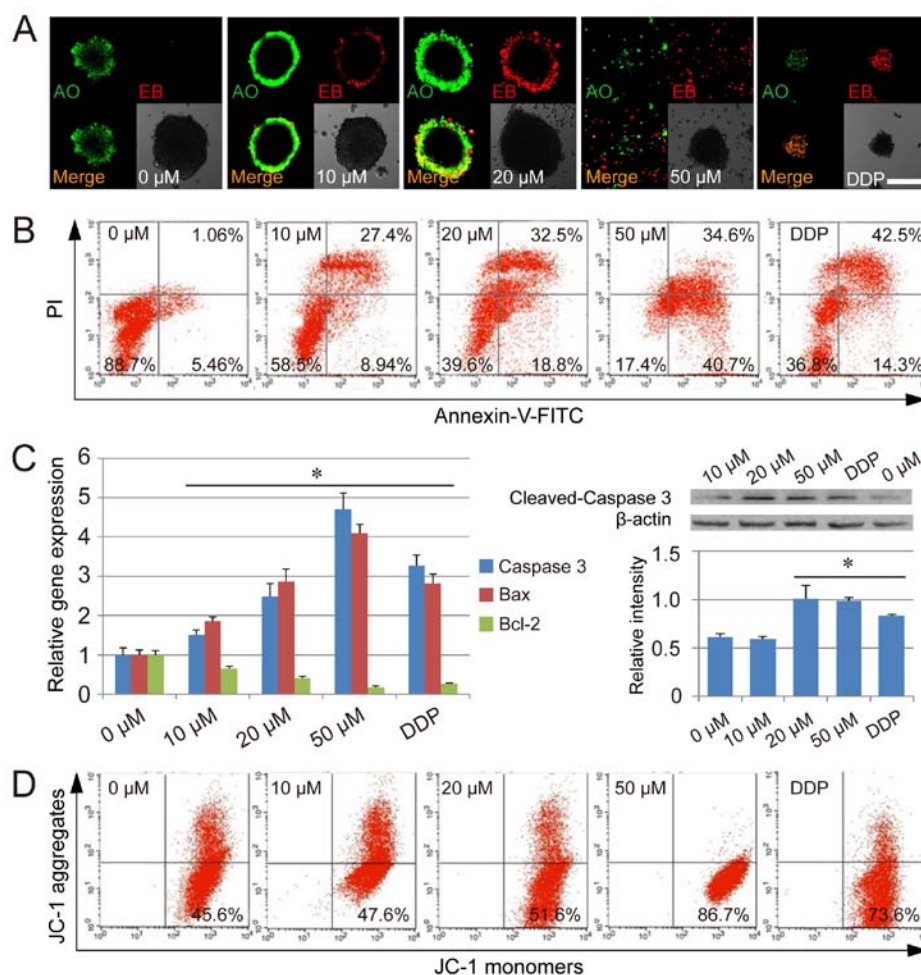


Figure 3. The effect of THIO on apoptosis in CSCs. (A) Results of AO/EB double-staining. After treatment with THIO at 0, 10, 20 and 50 μ M, the tumor spheres were stained with AO (red)/EB (green) and observed under confocal microscopy. Scale bar, 200 μ m. (B) Annexin V/PI double staining analysis of THIO-treated CSCs. Annexin V was labeled with FITC. The early-stage apoptotic cells (Annexin V-positive and PI-negative cells), as well as late-stage apoptotic cells (Annexin V-positive and PI-positive cells), in CSCs were analyzed with FACS. (C) Expression of apoptosis and anti-apoptosis genes and protein in CSCs treated with THIO. Relative gene expression results are shown on the left, while western blot results are shown on the right. Results are expressed as mean \pm SEM. A t-test was performed to compare the various groups and $P < 0.05$ was considered statistically significant. * $P < 0.05$ compared with the group of THIO at a concentration of 0 μ M. (D) THIO induces cell apoptosis in CSCs via the mitochondrial pathway. JC-1 staining was used to determine the loss of mitochondrial membrane potential in CSCs. Values in the right lower quadrant indicate the proportion of cells with loss of mitochondrial membrane potential.

CSCs could still form tumor sphere-like colonies when treated with THIO at 10 μ M. However, the number of colonies formed at 10 μ M was considerably lower than at 0 μ M. No colony formation could be observed at THIO concentrations of 20 μ M and above and after treatment with DDP (Fig. 2A).

In vitro invasion assays were performed using CSCs from HCT116 cells treated with THIO in a Transwell system. After crystal violet staining, we observed that the CSC invasion across the membrane from the upper chamber to the lower surface of the membrane was suppressed by THIO. The number of cells on the lower surface of the membrane clearly decreased in the THIO-treated groups at concentrations of 20 and 50 μ M, while a slight inhibition of the cell invasion ability was observed in the 10 μ M concentration group (Fig. 2B). After counting the number of cells on the lower surface of the membrane, we concluded that THIO could significantly suppress the invasion of CSCs ($P < 0.05$). This effect occurred in a concentration-dependent manner (52.0 \pm 8.0 cells for the 0 μ M group, 35.6 \pm 7.3 cells for the 10 μ M group, 22.0 \pm 5.4 cells

for the 20 μ M group, 10.3 \pm 4.0 cells for the 50 μ M group, and 14.3 \pm 3.3 cells for the DDP group) (Fig. 2B).

To further analyze the anticancer effect of THIO on CSCs derived from HCT116 cells, a CCK-8 assay was performed using treated cells (normal human lung fibroblast cell line, HELF cells, HCT116 cells and CSCs). The results indicated that the proliferation of HELF and HCT116 cells was significantly inhibited by THIO at 50 and 100 μ M, while that of CSCs was significantly inhibited by THIO at a concentration as low as 1 μ M. The inhibition ratio of THIO at 1 and 10 μ M was 19.2 and 39.5%, respectively. However, THIO concentrations of 20, 50 and 100 μ M exhibited inhibition at very high rates (94.5, 100 and 100%, respectively). The positive control group (10 μ M DDP) showed an inhibition rate of 82.7%. Interestingly, THIO at 20 μ M did affect the vitality of HELF cells, but the inhibition rate was only 10.6%, while that of CSCs was 94.5% (Fig. 2C). These results indicate that, at a suitable concentration, THIO may be an optimal novel agent for colon cancer treatment. However, further studies are necessary to elucidate the mechanism of action driving this anticancer activity.

Analysis of THIO-induced apoptosis in CSCs. THIO has been reported to induce cervical and endometrial cancer cell apoptosis (7). To detect whether THIO could induce apoptosis in CSCs derived from HCT116 cells, we carried out AO/EB staining. The CSC spheres were labeled by AO/EB after treatment with THIO at different concentrations, and dual staining was examined using confocal microscopy. No significant apoptosis was detected in the negative control group. However, in the group treated with THIO at a concentration of 10 μ M, many early-stage apoptotic cells (marked by yellow-green AO nuclear staining) and some late-stage apoptotic cells (marked by orange-red nuclear EB staining) were detected. At higher concentrations (20 and 50 μ M), the number of late-stage apoptotic cells was even higher (Fig. 3A). At 50 μ M, CSC spheres were much smaller than at lower concentrations, and many single cells could be observed in late-stage apoptosis (Fig. 3A). Further detection by the Annexin V/PI double staining assay revealed that THIO treatment could lead to an increase in the proportion of early-stage apoptotic cells (Annexin V-positive and PI-negative cells), as well as late-stage apoptotic cells (Annexin V-positive and PI-positive cells) in CSCs (Fig. 3B). Further analysis indicated that this induction of apoptosis by THIO occurred in a concentration-dependent manner. The percentage of early-stage apoptotic cells was 5.46, 8.94, 18.8, 40.7 and 14.3% at THIO concentrations of 0, 10, 20 and 50 μ M, and DDP treatment, respectively. The percentage of late-stage apoptotic cells was 1.06, 27.4, 32.5, 34.6 and 42.5% at THIO concentrations of 0, 10, 20 and 50 μ M, and DDP treatment, respectively (Fig. 3B).

To elucidate the mechanisms of cell apoptosis induced by THIO, cell apoptosis genes and mitochondrial membrane potential were assayed as part of this study. qPCR results indicated that apoptosis genes such as caspase-3 and Bax were significantly upregulated in THIO-treated CSCs ($P < 0.05$), while the anti-apoptosis gene Bcl-2 was significantly downregulated under the same conditions ($P < 0.05$) (Fig. 3C). Moreover, the expression of the caspase-3 gene was confirmed by the western blot analysis. To evaluate the THIO-induced cell apoptosis at the subcellular organelle level, JC-1 staining was carried out to determine the alterations in mitochondria. The percentage of cells with loss of mitochondrial membrane potential increased in a concentration-dependent manner (45.6% for the 0 μ M group, 47.6% for the 10 μ M group, 51.6% for the 20 μ M group, 86.7% for the 50 μ M group, and 73.6% for the DDP group) (Fig. 3D). The results suggest that THIO may induce apoptosis in CSCs from HCT116 cells via the loss of mitochondrial membrane potential.

Discussion

THIO has been used in human clinical studies for over 50 years, especially for tuberculosis (TB) therapy (22,23), alleviation of schizophrenia (24) and reduction of pain in cancer (25). In recent years, THIO has been reported to suppress cell proliferation and induce cell apoptosis in several types of cancers (2,7,9,26,27). However, the cytotoxic effect of THIO on colon cancer has not been examined, and the effects of THIO on the viability of CSCs, which are responsible for apoptosis resistance, self-renewal and differentiation, should

be investigated thoroughly. The present study revealed that THIO could reduce the viability of CSCs from colon cancer cells (HCT116) and induce apoptosis of CSCs via the mitochondrial pathway. Previous studies in our group revealed that THIO also induces apoptosis in CSCs derived from other cancer cell lines, such as DU145 (human prostate carcinoma cell line) (data not shown). Thus, THIO-based chemotherapy may prove useful in the treatment of various types of cancers. This must be evaluated in future clinical studies.

Although the anticancer effect of THIO has been demonstrated *in vivo* using mouse models (26,28,29), few clinical trials have been carried out in cancer patients (30) for the complex mechanisms of the THIO anticancer effect to be clearly understood. In the present study, JC-1 results showed that the mitochondrial membrane potential in CSCs was downregulated during apoptosis, which indicates that THIO-induced apoptosis in CSCs may be related to the mitochondrial pathway. In addition to the direct cytotoxic effect on cancer cells, THIO can potentially induce CSC differentiation to overcome neoplastic self-renewal, and compel CSCs to enter the normal cellular lifecycle, via antagonism of D2-family DRs differentially expressed in CSCs (15,31). However, this theory may only apply to a therapy targeting CSCs, and the effect of THIO on CSCs has so far been demonstrated only in human leukemia and breast cancer studies (15,16). Therefore, the specific effects of THIO on CSCs from other cancers, as well as the associated mechanisms, still require exploration.

THIO can prevent the exclusion of some small molecules from cancer cells (26,32,33). This may explain the anti-CSC ability of THIO that was discovered through small-molecule library screening (26,34). A previous study has indicated that THIO could reverse chemoresistance of cancer cells and achieve a significant therapeutic outcome in combination therapy with verapamil (35). A recent study also demonstrated that the combination of THIO and doxorubicin using polymeric micelles might provide a promising strategy for breast cancer treatment by targeting both cancer cells and cancer stem cells (16). Thus, THIO may be useful as a type of adjuvant in combination with other chemotherapeutic drugs.

In clinical studies of cancer, THIO is mainly used for managing depression and psychosis (30). Apart from understanding the therapeutic mechanisms, it is important to consider the optimal dose in order to avoid serious side-effects, which can include movement disorders and cardiac and central nervous system toxicity. Thus, the potential toxic effects of high-dose THIO treatments should be evaluated carefully.

Conclusion, this research showed that THIO could suppress proliferation, reduce invasion, and induce apoptosis in CSCs via the mitochondrial pathway in a concentration-dependent manner. Even though the anticancer effect of THIO still needs to be evaluated *in vivo*, and in well-designed human clinical trials, there is enough evidence to suggest that THIO may be a promising novel agent as an adjuvant for the treatment of colon cancer, and possibly other cancers.

Acknowledgements

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