BC-02 eradicates liver cancer stem cells by upregulating the ROS-dependent DNA damage

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Abstract. Cancer stem cells (CSCs) are responsible for chemoresistance, tumor recurrence and metastasis. Reportedly, aminopeptidase N (APN, also known as CD13) is a marker for semi-quiescent CSCs and a therapeutic target in human liver CSCs. In the present study, the effect of BC-02, a compound obtained by conjugating a CD13 inhibitor bestatin and fluorouracil (5-FU), was investigated toward liver CSCs. Tumor spheres formed in serum-free culture conditions have been successfully used to enrich CSCs. In this study, the sphere cells were shown to have several characteristics of CSCs, including drug resistance, high tumorigenicity, epithelial-mesenchymal transition (EMT) phenotype, lower reactive oxygen species (ROS) levels, greater colony-forming efficiency and increased proliferation capacity in vitro. Furthermore, BC-02 effectively suppressed self-renewal and malignant proliferation of CSCs compared with 5-FU, bestatin, and even the combination of 5-FU and bestatin. In addition, cell proliferation was effectively suppressed when exposed to 5-FU plus CD13-neutralizing antibody (CD13 Ab) compared with 5-FU alone. BC-02 can effectively inhibit the activity of CD13. Results demonstrated that CD13 inhibitor BC-02 impaired the properties of liver CSCs by targeting CD13 and upregulating the intracellular ROS and ROS-induced DNA damage. BC-02 might be a potential therapeutic agent for eradicating the liver CSCs and overcoming chemoresistance in liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide, and its morbidity and mortality rates have been increasing in recent years (1,2). Currently, systemic therapies and anticancer drugs for HCC have obtained considerable advances; however, the incidence rate of resistance to chemotherapy, tumor recurrence and metastasis is still increasing (3,4). Accumulating evidence has revealed that one of the reasons for the resistance to anticancer drugs is the existence of CSCs or tumor-initiating cells (TICs) (5).

The concept of CSCs or TICs was first proposed approximately 50 years ago (6). The existence of CSCs has been demonstrated in leukemia (7) and solid tumors, including breast, colon, brain and liver cancers (8-13). CSC is a small subpopulation of cancer cells with stem cell properties, such as self-renewal, pluripotency, chemoresistance and limitless proliferation (14). Substantial evidence revealed that CSCs are responsible for initiation and resistance to chemotherapy; and the cancer always relapses and metastasizes due to the persistence of CSCs (14,15). Hence, inhibiting CSC proliferation or even eradicating them may be a pivotal strategy for overcoming chemoresistance and improving the curative effect in liver cancer.

To better investigate CSC properties and novel anticancer drugs, many studies have been created in vitro model system using serum-free stem cell conditional medium to select and expand CSCs. Tumor spheres formed in this condition possess cancer stem cell characteristics, including self-renewal, proliferation, chemoresistance and higher tumorigenicity. Thus, they are considered to be CSCs (16,17). This sphere cell
formation method has been successfully used to enrich CSCs from liver, brain, lung and ovarian cancers (17-20). Thus, this method was also used to enrich CSCs, and the condition of the culture medium was optimized to create a more suitable microenvironment for better forming liver cancer stem cells (LCSCs).

CD13/APN is a marker for semi-quiescent CSCs and a therapeutic target in human liver CSCs (5). CD13 is a zinc-binding type 2 transmembrane ectopeptidase (150 kDa), which is related to malignant behavior in many cancers, such as prostate, colon and lung cancers (21-23). In in vivo transplantation model of mice, CD13+ cells survived, and the expression of CD13 in vivo is related to malignant behavior in many cancers, such as prostatic, colon and lung cancers (21-23). In in vivo transplantation model of mice, CD13+ cells survived, and the expression of CD13 is upregulated after 5-FU treatment alone, which is one of the most common cytotoxic drugs in HCC treatment.

Thus, co-processing with a CD13 inhibitor could be helpful for radical elimination of CD13+ cells or liver CSCs (24). Bestatin (ubenimex) is reported to be a CD13 inhibitor, which is constantly used for adult acute nonlymphocytic leukemia (25). As expected, combining bestatin with 5-FU treatment efficiently elicits tumor regression and improves the effect of liver cancer treatment (5). Thus far, few drugs, which could not only target liver CSCs but has cytotoxic effects on the CSCs, may kill CSCs and reveal a promising cure for liver cancer.

In the present study, the serum-free stem cell conditional medium was optimized and sphere cells were successfully enriched and expanded, which possessed the characteristics of stem cells. The compound BC-02, an APN/CD13 inhibitor, showed a significant effect on self-renewal and malignant proliferation of liver CSCs compared to the control, bestatin, 5-FU and even 5-FU plus bestatin. Subsequently, BC-02 could target CD13 and increase the intracellular ROS and the ROS-induced DNA damage, thereby inhibiting the liver CSCs and overcoming the chemoresistance in liver cancer. Therefore, BC-02 might be a novel therapeutic drug for improving the survival rate of liver cancer patients.

Materials and methods

Chemicals and reagents. Bestatin (Fig. 1A) and 5-FU (Fig. 1B) were obtained from Shanghai Biochempartner Co., Ltd. (Shanghai, China). The compound BC-02 (Fig. 1C) was synthesized by conjugating a CD13 inhibitor bestatin and 5-FU in the Department of Medicinal Chemistry, School of Pharmacy, Shandong University (26). The BC-02 could be decomposed into 5-FU and bestatin, when it was preliminary analysed by electrospray ionization mass spectrometry (ESI-MS) (Fig. 2). 5-FU, bestain and BC-02 were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at 400 mM as stock solution. The 5-FU and bestatin was mingled by equimolar concentration at 800 mM as stock solution, it is represented by 1:1 in the subsequent experiments. The CD13 mouse anti-human antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Methyl thiazolyl tetrazolium (MTT) was purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China).

Cell culture. Human liver cancer cell lines PLC/PRF/5 and Huh7 were purchased from the Cell Bank of Shanghai (Shanghai, China) and cultured in modified Eagle’s medium (MEM) and Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies Corp., Carlsbad, CA, USA) respectively, both supplemented with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel). The cells were cultured at 37˚C in a humidified 5% CO2 incubator and passaged every other day.

Sphere culture and self-renewal assay. PLC/PRF/5 and Huh7 parental cells were collected and washed with phosphate buffered saline (PBS) to remove serum, respectively. Then the cells were plated in serum-free stem cell conditional medium composed of DMEM/F12 (HyClone Laboratories, Inc., South Logan, UT, USA), KnockOut™ serum replacement, 2% B-27 supplement, 1% N-2 supplement (Gibco/Invitrogen, Grand Island, NY, USA), 20 ng/ml recombinant human insulin-like growth factor-I (IGF-I), 20 ng/ml animal-free recombinant human epidermal growth factor (EGF), 10 ng/ml recombinant human fibroblast growth factor-basic (b-FGF), (PeproTech, Rocky Hill, NJ, USA), 2 mM L-glutamine, 5 µg/ml heparin sodium and 1% penicillin-streptomycin (Beijing Solarbio Science and Technology). The cells were suspended in ultralow attachment 6-well plates (Corning Inc., Corning, NY, USA) at a density of 10,000 cells/well. When the spheres grew to 100-200 µm in diameter, the spheres were dissociated with trypsin-EDTA. Then the single cells were re-suspended in stem cell conditional medium to re-form spheres and the medium was changed every other day. The PLC/PRF/5 spheres were passaged every 5-7 days. The Huh7 spheres were passaged every 3-5 days. In addition, the isolated PLC/PRF/5 parental cells were cultured in ultralow attachment 6-well plates at a density of 2,000 cells/well for 48 h. Then cells were treated with 400 µM various chemicals (5-FU, bestatin, 1:1, BC-02, and BC-02 + NAC) for 2 h. Cells were pretreated by 500 µM NAC for 2 h. Chemicals were removed after 6 h. After further culturing for 3 days, the spheres were analyzed by optical microscopy.

Colony formation assay. PLC/PRF/5 parental and sphere cells were dissociated into single cells and the cells were seeded in MEM with 10% FBS on 6-well plates (1,000 cells/well). After 8-10 days, the cells grew to visible colonies (>50 cells). The colonies were counted under an ordinary optical microscope. The colonies were analyzed by optical microscopy.
for 6 h. Cells were pretreated by 500 µM NAC for 2 h. Then cell medium was changed to remove chemicals. The colonies were counted as mentioned and PLC/PRF/5 sphere cells were treated with 400 µM drugs, and the concentration of chemicals for Huh7 sphere cells was 200 µM.

Western blot analysis. Parental and sphere cells were collected, washed with PBS, and lysed in RIPA buffer with protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were measured using a BCA protein assay kit (Beijing Solarbio Science and Technology). Then quantified proteins were electrophoresed, separated in SDS-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore). Rabbit anti-human N-cadherin antibodies (dilution 1:1,000; Abcam, Cambridge, MA, USA), rabbit anti-human vimentin (dilution 1:1,000; Cell Signaling Technology, Danvers, MA, USA), and mouse anti-human beta actin (dilution 1:1,000; PeproTech) were used as the primary antibodies, and horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin (dilution 1:5,000; Beyotime Institute of Biotechnology, Shanghai, China) were used as the secondary antibody. Blots were acquired by enhanced chemiluminescence kit (Millipore).

MTT assays. Cell viability was measured by the MTT assay. PLC/PRF/5 parental and sphere cells were dissociated into single cells. The dissociated parental cells were re-suspended in MEM with 10% FBS on 96-well plates at a density of 5,000 cells/well, and the dispersed sphere cells were re-suspended in serum-free stem cell conditional medium on ultralow attachment 96-well plates (5,000 cells/well). After 24 h, the cells were treated with various concentrations of compounds. Two days after treatment, cells were incubated with MTT solutions for 4 h at 37˚C. Then the DMSO was added to each well. After that, the absorbance was measured at 570 nm using a multi-functional microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA). Huh7 cells were treated with the same method. The effects of CD13 inhibition on cell proliferation of spheres were examined using this method.

Cell proliferation in vitro was also measured by MTT method. The enzymatically dissociated cells were seeded in ultralow attachment 96-well plates in 200 µl of serum-free stem cell conditional medium at a density of 1,000 cells/well. Cells were fed with stem cell conditional medium every day. The absorbance were measured every 12 h for 6 consecutive days at 570 nm using a multi-functional microplate reader and the growth curves were draw using the data.

Enzyme activity assay. The APN activity was determined as previously described (24). PLC/PRF/5 cells were re-suspended in PBS in a 96-well plate with specified concentrations of bestatin or BC-02. Cells were incubated with L-leucine-p-nitroanilide (Sigma-Aldrich) for 30 min at 37˚C. Then the enzyme activity was obtained by measuring the absorbance at 405 nm using a multi-functional microplate reader. The APN enzyme activity inhibition rates were calculated by (OD control - OD tested)/OD control x 100%.

Cellular ROS detection. Parental and sphere cells were trypsinized with trypsin-EDTA and the isolated cells were incubated at 37˚C for 30 min in darkness with 2,7'-dichlo-
rofluorescein diacetate (DCFH-DA). After washing with PBS, the fluorescence intensity of DCF inside the cells was measured using a FACSCan flow cytometer. Intracellular ROS levels were analyzed using FlowJo 7.6 software and the data represented the mean fluorescence intensity of 30,000 events.

Alkaline comet assay. To determine the oxidative DNA damage, the alkaline comet assay was performed according to the manufacturer's instructions. Briefly, PLC/PRF/5 sphere cells were treated with various drugs (400 µM of 5-FU, bestatin, 1:1, BC-02 and BC-02 + NAC) for 12 h. Cells were pretreated by 500 µM NAC for 2 h. Then the cells were dissociated into single cells. The resulting cells were mixed with 0.7% low-melting agarose, added to slides pre-coated with 0.55% normal-melting agarose, covered with coverslips and allowed to solidify at 4˚C for 30 min. Then the coverslips were gently removed and the slides were kept in lysis buffer (2.5 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH 10.0) for 1.5 h, submerged in alkaline electrophoresis solution (10 M NaOH, 0.2 M EDTA, pH >13) for 50 min, and subjected to electrophoresis in the same buffer at 25 V for 25 min. After electrophoresis, cells were washed with neutral buffer (pH 7.5) and stained with propidium iodide (PI) for 10 min. The images were taken with a fluorescent microscope and the comets were quantified by determination of the percentage of DNA in the tail using TriTek Comet Score™ Freeware v1.5. Huh7 cells were treated with the same method and the concentration of the chemicals was 200 µM.

Nude mouse tumor formation assay. Four to five week-old female nude mice were purchased from the Hunan SJA Laboratory Animal Co., Ltd., (Hunan, China) and maintained in a specific pathogen-free conditions. All animal experiments were approved by the Guidelines of the Animal Care and Use Committee at Weifang Medical University. The defined number of viable parental and sphere cells was subcutaneously injected into the left or right flank of mice. After the inoculation, tumor formation and growth were monitored every day and mice were sacrificed at 12 weeks.

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<th>No. of cells injected</th>
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Statistical analysis. Each experiment was performed at least three times, and quantitative data were expressed as mean ± standard deviation (SD). Statistical differences between the two groups were evaluated by the Student's t-test using the SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). P<0.05 was defined as significant.

Results

PLC/PRF/5 and Huh7 cells form suspended spheres. Previous studies demonstrated that the spheres, which are suspended culture of cancer cells with growth factors in serum-free medium, are enriched with CSCs. To successfully induct CSCs, a more suitable culture system was first determined. Through careful screening, the suitable culture, which contains DMEM/F12, serum replacement, B-27 supplement, N-2 supplement, IGF-1, EGF, b-FGF, L-glutamine, heparin sodium and penicillin-streptomycin, was determined. In the culture system, two human HCC cell lines, namely, PLC/PRF/5 and Huh7, successfully formed suspended spheres. The status of the spheres was stably maintained even after several generations, indicating their capability for self-renewal similarly to TICs. Fig. 3 shows that cells under conventional medium with 10% FBS formed epithelial morphology, and the images of cells cultured in the serum-free stem cell conditional medium formed suspended, self-renewing spheres, which were observed by a microscope.

PLC/PRF/5 and Huh7 sphere cells possess the characteristics of CSCs. Both PLC/PRF/5 and Huh7 spheres possessed the characteristics of CSCs, including drug resistance, high
tumorigenicity, EMT phenotype, lower ROS levels, greater colony-forming efficiency, and increased proliferation capacity in vitro. MTT assays were performed to examine the sensitivity of spheres and parental cells to our chemotherapy drugs. Spheres and parental cells of liver cancer cell lines, such as PLC/PRF/5 and Huh7, were treated with 5-FU, bestatin, 1:1, and BC-02, respectively, with different concentrations for 48 h. As shown in Fig. 5A and B, the spheres showed much lower inhibition effect of the chemotherapy drugs than their parental cells, which indicates that spheres had increased chemoresistance. Tumorigenicity in vivo was analyzed by tumor formation assay using nude mice. As shown in Table I, 1x10^5 sphere cells derived from PLC/PRF/5 cells successfully formed tumors in one out of five mice, whereas similar number of PLC/PRF/5 parental cells failed to form any tumors. Furthermore, although the number of parental cells increased to 1x10^6, there was no tumor generated. In addition, 1x10^6 sphere cells were capable of forming tumors in three out of five mice. This result indicated that the sphere cells possessed high tumorigenicity in vivo and increased CSC properties. Recently, EMT has been considered to be associated with CSC stemness characteristic. EMT-related regulatory proteins, including N-cadherin and vimentin, were examined by western blotting. As shown in Fig. 4A, the mesenchymal markers (N-cadherin and vimentin) were upregulated in PLC/PRF/5 and Huh7 spheres compared with their parental cancer cells. Cancer stem cells which are similar to stem cells normally possess low levels of intracellular ROS. Thus, flow cytometric analyses were performed to examine intracellular ROS levels. Intracellular ROS levels both in PLC/PRF/5 and in HuH7 were lower in sphere cells than in parental cells (Fig. 4D-F). Colony formation and cell proliferation assays were also performed to evaluate the proliferative ability in vitro of the PLC/PRF/5 sphere and parental cells. As shown in Fig. 4B, more clone numbers of the sphere cells were significantly formed compared with the parental cells, and the proliferation of the PLC/PRF/5 sphere cells was...
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Figure 5. Effects of BC-02 on self-renewal and proliferation of sphere cells. (A) PLC/PRF/5 cell line. (B) Huh7 cell line. Spheres and parental cells were exposed to various concentrations of chemotherapy drugs for 48 h. Cell vitality was determined by the MTT assay. Bars represent the mean of three determinations ± SD. (C) Sphere formation assay of PLC/PRF/5 cell line was performed. Spheres were cultured in the absence or presence of 400 µM different chemotherapy drugs for 6 h. BC-02 markedly inhibited the number and size of PLC/PRF/5 spheres. Bright-field images are shown. Scale bar, 100 µm. (D) PLC/PRF/5. (E) Huh7. The clonogenic ability of sphere cells stimulated with various agents was examined using colony formation assay. Representative examples are provided. (F) Statistical analysis of (D). Three independent experiments were performed and the data represent the mean ± SD ("P<0.01 vs. 5-FU or 1:1, ##P<0.01 vs. BC-02). (G) Statistical analysis of (E). Three independent experiments were performed and the data represent the mean ± SD (##P<0.01 vs. BC-02).

faster than that of their parental cells (Fig. 4C). These results supported the greater proliferative ability in vitro.

BC-02 effectively inhibits proliferation and self-renewal of liver cancer stem cell. Spheres cultured in stem cell conditional medium have been proven to possess the characteristics of CSCs and to enrich the CSCs. Then, the effect of BC-02 on PLC/PRF/5 or Huh7 spheres in vitro assays was examined. Both cell lines, namely, PLC/PRF/5 and Huh7, were used to perform cell proliferation assay. BC-02 significantly suppressed the proliferation not only of parental cells but also sphere cells compared with other chemotherapy drugs in a dose-dependent manner (Fig. 5A and B). Furthermore, sphere formation experiments of PLC/PRF/5 cell line were performed using different chemotherapy drugs, such as 5-FU, bestatin, 1:1 and BC-02, with similar concentrations. As shown in Fig. 5C, BC-02 markedly inhibited the number and size of tumor spheres compared with the control, bestatin, 5-FU and even 1:1 groups. In addition, BC-02 also effectively impaired the formation of colonies compared with other groups not only of PLC/PRF/5 sphere cells but also of Huh7 sphere cells (Fig. 5D-G). Overall, these results indicate that
BC-02 effectively suppresses malignant proliferation and self-renewal of CSCs. The co-treatment of sphere cells with the ROS scavenger NAC, which is an antioxidant, attenuated the inhibitory effect on proliferation, self-renewal and clone formation induced by BC-02. Those results prompted that lower level of ROS may be a protection for cells and BC-02 may damage the cells by increasing the intracellular ROS.

BC-02 prevents the properties of hepatoma stem cells by targeting CD13 and increasing the ROS-dependent DNA damage. CD13 is a therapeutic target in human LCSCs. The effect of CD13 inhibition on cell proliferation was confirmed by cell proliferation assay in PLC/PRF/5 and Huh7 sphere cells. Cell proliferation was effectively suppressed after exposure to 5-FU plus CD13 Ab compared with 5-FU alone (Fig. 6A and B). In addition, bestatin was recognized as CD13 inhibitor, which specifically blocks CD13 and antagonizes with the zinc-binding site of the CD13 domain. The inhibitory effects of bestatin and BC-02 on CD13 activity were measured using PLC/PRF/5 cells, which had high expression levels of CD13. As shown in Fig. 6C, CD13 activity was inhibited by bestatin as reported. BC-02 even showed higher inhibition rate than bestatin in a concentration-dependent manner. These results indicated that BC-02 was more effective to target CD13.
the prognosis of patients with HCC were indispensable. In this study, BC-02 can potently inhibit the self-renewal and proliferation of liver CSCs by targeting CD13 and increasing the intracellular ROS and the ROS-induced DNA damage.

PLC/PRF/5 and Huh7 spheres formed and acquired stemness in the serum-free medium. The sphere cells had several characteristics of CSCs, including drug resistance, high tumorigenicity in nude mice, EMT phenotype, lower ROS levels, greater colony-forming efficiency, and increased proliferation capacity in vitro. Similar to previous studies, spheres possessed the stemness that was expanded by sphere culture methods (27-30). What was interesting was that the proliferation of the Huh7 sphere cells was slower than their parental cells (data not shown), which may be because the culture medium was not completely appropriate to the Huh7 cell line, and the cells could not enter a rapid proliferation period. Thus, different CSCs had different microenvironments that should be adaptive to their survival. Efforts are still necessary for exploring more suitable CSC media for various cell lines.

5-FU is one of the most common anticancer drugs in HCC treatment. It has toxicity to the hematopoietic function of bone marrow (31,32). Bestatin is a recognized CD13 inhibitor that is constantly used as an adjuvant, which can enhance the immunity and prolong the lifetime of patients with acute adult non-lymphocytic leukemia. Studies have reported that the combination therapy of 5-FU plus bestatin efficiently enhanced the antitumor effects of liver cancer (5,33). In this study, BC-02 was synthesized with the 5-FU and bestatin monomer. BC-02 could target CD13 and enter the cells accurately with cytotoxic effects. Thus, it might reduce the drug side-effects by cutting down the dosage and increasing the efficacy of chemotherapeutics. BC-02 can effectively inhibit the activity of CD13 compared with bestatin in a dose-dependent manner. Therefore, BC-02 is a potential molecular targeting agent to CD13. BC-02 also can slowly release bestatin and 5-FU (Fig. 2). Thus, BC-02 which is similar to 5-FU can possibly exploit the advantages of cytotoxicity to cancer cells. As expected, BC-02 effectively suppressed the number and size of tumor spheres, the formation of colonies, and the malignant proliferation of the CSCs compared with 5-FU, bestatin, and even the combination 5-FU with bestatin. In brief, the newfangled CD13 inhibitor BC-02 could efficiently inhibit self-renewal and proliferation of liver CSCs. Experiments in vivo must be conducted for further research on this novel antineoplastic agent.

Previous findings indicated that CSCs could always resist chemotherapy drugs and maintain long-term survival. Evidence showed that those characters of CSCs are related to cell cycle dormancy, lowering the ROS levels in CSCs, and DNA repair mechanisms (34). In the present study, the spheres of PLC/PRF/5 and Huh7 contained lower intracellular ROS levels than their parental cells. Previous studies suggested that chemotherapeutic drugs were used to cause DNA damage through ROS accumulation (35), and the combination therapy of 5-FU plus bestatin efficiently enhanced the antitumor effects by upregulating intracellular ROS levels and inducing DNA injury (5,33). Whether generating similar processes of liver CSCs after exposure to BC-02 was examined. As expected, BC-02 effectively inhibited self-renewal and proliferation of CSCs, increased the intracellular ROS and promoted the

Discussion

CSCs with stem cell properties are a minor population of cancer cells. Their presence is responsible for the chemoresistance to the systemic chemotherapy in HCC. Therefore, searching for a novel agent that can target CSCs and exhibit cytotoxic effects on CSCs for eradicating liver CSCs and improving

Figure 7. The mechanism behind the effects of BC-02 on the liver CSC proliferation. BC-02 could target CD13 and cause an increase in intracellular ROS and ROS-induced DNA damage.
DNA oxidative damage. The treatment of CSCs with NAC recovered the proliferation of CSCs, canceled the increase of the ROS level and repaired the DNA damage. The molecular mechanism of BC-02 to CSCs was consistent with previous studies. However, further studies are required to determine the molecular mechanism of increasing production of intracellular ROS or reducing the scavenger of ROS when CSCs are exposed to BC-02.

A recent series of studies provided further evidence that cancer progress is dependent not only on the malignant cells but also on the microenvironment. This study demonstrated that glioblastoma, one of the malignant brain tumors, maintains their CSCs in vascular niches. Targeting cancer stem cell niches may be a powerful approach for the treatment of cancer (36). Recent data showed that anti-angiogenic strategies combined with conventional cytotoxic drugs reduce the tumor stem-like cell fraction (37). Sorafenib is always used in the systemic treatment of HCC, and it is the inhibitor of the receptors of the anti-angiogenic multi-kinase. Thus, BC-02 combined with sorafenib might effectively provide curative effect for patients with HCC.

In conclusion, we indicated that combination of BC-02 may efficiently inhibit the self-renewal and proliferation of liver CSCs compared with 5-FU, bestatin, and even the combination of 5-FU and bestatin. Furthermore, as summarized in Fig. 7, BC-02 was able to target CD13 and then increase the intracellular ROS and the ROS-induced DNA damage. This will lead to the inhibition of self-renewal and induction of apoptosis of LCSCs. Therefore, the novel CD13 inhibitor BC-02 may be a potential strategy for eradicating liver CSCs and overcoming chemoresistance in liver cancer.

Acknowledgements

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