Tumor Biol. (2014) 35:9563–9569
DOI 10.1007/s13277-014-2233-1

RESEARCH ARTICLE

Inhibitory effects of sea buckthorn procyanidins on fatty acid synthase and MDA-MB-231 cells

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Received: 21 May 2014 / Accepted: 12 June 2014 / Published online: 24 June 2014

Abstract Fatty acid synthase (FAS) is overexpressed in many human cancers including breast cancer and is considered to be a promising target for therapy. Sea buckthorn has long been used to treat a variety of maladies. Here, we investigated the inhibitory effect of sea buckthorn procyanidins (SBPs) isolated from the seeds of sea buckthorn on FAS and FAS overexpressed human breast cancer MDA-MB-231 cells. The FAS activity and FAS inhibition were measured by a spectrophotometer at 340 nm of nicotinamide adenine dinucleotide phosphate (NADPH) absorption. We found that SBP potently inhibited the activity of FAS with a half-inhibitory concentration (IC_{50}) value of 0.087 μg/ml. 3-4,5-Dimethylthiazol-2-yl-2,3-diphenyl tetrazolium bromide (MTT) assay was used to test the cell viability. SBP reduced MDA-MB-231 cell viability with an IC_{50} value of 37.5 μg/ml. Hoechst 33258/propidium iodide dual staining and flow cytometric analysis showed that SBP induced MDA-MB-231 cell apoptosis. SBP inhibited intracellular FAS activity with a dose-dependent manner. In addition, sodium palmitate could rescue the cell apoptosis induced by SBP. These results showed that SBP was a promising FAS inhibitor which could induce the apoptosis of MDA-MB-231 cells via inhibiting FAS. These findings suggested that SBP might be useful for preventing or treating breast cancer.

Keywords Fatty acid synthase · Inhibitor · Sea buckthorn · Procyanidins · MDA-MB-231 cells · Cell apoptosis

Introduction

Sea buckthorn (*Hippophae rhamnoides* L.), belonging to the family Elaeagnaceae, is a winter-hardy, deciduous shrub indigenous to Asia and Europe [1]. It can be found growing widely in the northern and western regions of China, locally known as "Shaji." Recently, it has been extensively planted across Northern China and other countries, to prevent soil erosion and to serve as an economic resource for food and medicine products. Sea buckthorn is known as a functional food for its healing properties, nutritive values, and therapeutic applications [2]. The seeds, berries, leaves, and bark of sea buckthorn have been used for hundreds of years in China for medicinal and nutritional purposes [3–6]. Both the flavonoids and the oils from sea buckthorn have several potential applications [7]. Therapeutic efficacy has been demonstrated in gastric ulcers, liver injury, wound healing, atopic dermatitis, and cardiovascular diseases [8–15].

The literature describing the role of sea buckthorn in the prevention and control of cancer is limited, however, certain analysis of the known experimental research information on anticancer by sea buckthorn available at present [16]. The inhibition of sea buckthorn oil on the cancer cells have been reported [17]. Sea buckthorn oil was found to enhance nonspecific immunity and to provide antitumor effects in preliminary laboratory study [18]. Sea buckthorn berry was found to inhibit cancer cell proliferation and suppression [19]. Flavonoids isolated from sea buckthorn were found to have in vitro antitumor activity against Bcap-37 and BEL-7402 cells [20, 21]. It was reported that 70 % EtOH extracts of sea buckthorn inhibited tumor-promoter-induced inflammation in mice [22]. However, the mechanisms involved remain unclear.

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Fatty acid synthase (FAS, EC 2.1.3.85), a multifunctional enzyme that catalyzes fatty acid palmitate synthesis from acetyl-CoA (Ac-CoA), malonyl-CoA (Mal-CoA), and nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, is a key enzyme for the de novo long-chain fatty acid biosynthesis. It was reported that human cancer cells express high levels of FAS and undergo significant endogenous fatty acid synthesis [23]. FAS is a key enzyme involving membrane formation and energy metabolism which is significant in tumor cells for rapid proliferation [24–26]. Some studies show that FAS inhibitors such as C75, cerulenin, and epigallocatechin gallate (EGCG) can induce cancer cell apoptosis specifically [27–29]. Therefore, FAS, as an attractive antitumor target, relates to survival of the cancer cells, and its inhibitor might be a potential antitumor drug.

In the present study, we investigated the effect of sea buckthorn procyanidins (SBPs), as a novel FAS inhibitor, on FAS overexpressed human breast cancer MDA-MB-231 cells. We demonstrated that SBP induced MDA-MB-231 cells apoptosis by inhibiting intracellular FAS activity.

Materials and methods

Reagents

Ac-CoA, Mal-CoA, NADPH, dimethyl sulfoxide (DMSO), Hoechst 33258, and annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). MDA-MB-231 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Beijing, China). 3-4,5-Dimethylthiazol-2-yl-2,3-diphenyl tetrazolium bromide (MTT), PBS, and the TRIzol reagent were purchased from Invitrogen (Beijing, China). Ethanol was a local product of analytical grade.

Preparation of FAS and its substrate

The preparation of FAS from chicken liver was performed as described previously [30]. The amino acid sequence of human FAS has 63 % identity with the sequences of chicken enzymes [31]. The purified FAS were homogenized by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate, respectively. The concentrations of FAS and its substrates were determined by UV-Vis spectrophotometer (Amersham Pharmacia Ultrospec 4300, England, UK) using the following experimental parameters: FAS, 4.83×10^5 M^{-1} cm^{-1} at 279 nm; Ac-CoA, 1.54×10^4 M^{-1} cm^{-1} at 259 nm, pH 7.0; Mal-CoA, 1.46×10^4 M^{-1} cm^{-1} at 260 nm, pH 6.0; acetoacetyl-CoA, 1.59×10^4 M^{-1} cm^{-1} at 259 nm, pH 7.0; NADPH, 6.02×10^3 M^{-1} cm^{-1} at 340 nm, and 1.59×10^4 M^{-1} cm^{-1} at 259 nm, pH 9.0.

Preparation of SBP from sea buckthorn

Dried powder of sea buckthorn seeds (500 g) was extracted with 70 % ethanol (1:10 *m*/*v*) for three times at a temperature of 50 °C. The extract was filtered and concentrated under reduced pressure, which yielded 500-ml aqueous solution. Two thousand milliliters of anhydrous ethanol was added to this solution. After stirring for 20 min, the extract was placed on a refrigerated stand for more than 2 h, centrifuged to remove precipitate, and the supernatant was collected and concentrated under reduced pressure at a temperature of 55 °C to 100 ml. This concentrated solution was loaded and absorbed by AB-8 macroporous resin and eluted with water, 50 % ethanol, and 70 % ethanol, sequentially. Fifty percent ethanol eluent was collected and concentrated under reduced pressure at a temperature of 55 °C to yield dried procyanidins of 5 g. Procyanidin concentration of 51.1 % was determined by ammonium ferric sulfate assay.

Assays of FAS activity

The FAS activity was measured at 37 °C by a spectrophotometer at 340 nm of NADPH absorption. The overall reaction system contained 100 mM KH_2PO_4-K_2HPO_4 buffer, 1 mM EDTA, 1 mM dithiothreitol, 3 μM Ac-CoA, 10 μM Mal-CoA, 35 μM NADPH, and 10 μg FAS in a total volume of 2 ml as previously described [30, 32].

Assays of FAS inhibition

To examine the fast-binding reversible inhibition effect of SBP on FAS, SBP was added to the assay system before FAS initiated the reaction. The activities of FAS with and without SBP were represented as A_i and A_0. The value of $A_i/A_0\times100$ % was the residual activity of FAS. The half-inhibitory concentration (IC_{50}) was calculated from the plot of residual activity versus SBP concentration with Origin 7.5 program. Dissolution of SBP was lower in 0.5 % (*v*/*v*) DMSO, while FAS activity was not affected by this concentration of DMSO (data not shown).

Cell growth inhibition assay

Tests were performed in 96-well plates. MDA-MB-231 cells were cultured in the plates until confluence, and the cells were incubated with either DMSO (1:1000) or increasing concentrations of SBP for 24 h (37 °C, 5 % CO_2). The medium was then changed to a fresh one within 0.5 mg/ml MTT. After 4-h incubation at 37 °C, the plates were again decanted, and 150 μl of DMSO was added to solubilize the formazan

crystals present in viable cells. The plate was analyzed by spectrometry at 492 nm by a microplate spectrophotometer (Multiskan, MK3). The wells containing no cells served as a background for the assay. Data were obtained from the average of five experiment wells, and the assay was repeated three times.

Hoechst 33258/PI dual staining

MDA-MB-231 cells were seeded in six-well culture dishes (2.5×10^6 cells/well). After experimental treatment, the cells were washed twice with PBS and stained with Hoechst 33258 (5 μg/ml) for 10 min, 37 °C in the dark, and then stained with PI for 10 min, at 4 °C, and finally followed by extensive washes. Nuclear staining was examined under the fluorescence microscope, and images were captured using Image-Pro Plus software (Media Cybernetics, Silver spring, MD).

Detection of cell apoptotic rates by flow cytometry

Apoptosis was determined by staining cells with annexin V FITC and PI labeling. Briefly, 1.5×10^5 cells/ml were incubated with or without SBP (10, 20, and 30 μg/ml) for 6 h. Afterward, the cells were washed twice with ice-cold PBS, and then, annexin V-FITC and PI were then applied to stain cells as the kit directions. The status of cell staining was analyzed by using flow cytometer (Becton Dickinson). Viable cells were negative for both PI and annexin V-FITC; apoptotic cells were positive for annexin V-FITC and negative for PI, whereas late apoptotic dead cells displayed strong annexin V-FITC and PI labeling. Non-viable cells, which underwent necrosis, were positive for PI but negative for annexin V-FITC.

Cell FAS activity assay

FAS activity in cells was assessed as described previously with some modifications [33]. In brief, after the cells were harvested, pelleted by centrifugation, resuspended in cold assay buffer (100 mM potassium phosphate buffer, 1 mM EDTA, 1 mM PMSF, and 1 mM dithiothreitol, pH 7.0), ultrasonically disrupted, and centrifuged at 12,000 rpm for 30 min at 4 °C, the supernatant was collected for the overall reaction assay. Fifty-microliter supernatant was added to the reaction mix containing 25 mM KH_2PO_4-K_2HPO_4 buffer, 0.25 mM EDTA, 0.25 mM dithiothreitol, 30 μM Ac-CoA, 100 μM Mal-CoA, and 350 μM NADPH (pH 7.0) in a total volume of 200 μl. Protein content in the supernatant was determined using a bicinchoninic acid (BCA) assay (Pierce), and results were expressed as the specific activity of FAS (U/mg).

Effect of sodium palmitate on MDA-MB-231 cells treated with SBP

MDA-MB-231 cells were cultured in the 96-well plates until confluence, and the cells were incubated with either increasing concentrations of SBP (0, 25, and 50 μg/ml) or 0, 25, and 50 μM sodium palmitate at 37 °C for 24 h in a humidified CO_2 incubator. The cell viability was measured by the MTT assay, which was performed according to the above instructions.

Results

Inhibition of SBP on FAS activity

The reversible inhibition of FAS on the activity by SBP was determined. As shown in Fig. 1, the activity of FAS was decreased following the increase of SBP concentration. A concentration of 0.087 μg/ml of SBP inhibited 50 % of the overall reaction activity of FAS.

SBP inhibited the viability of MDA-MB-231 cells in vitro

To identify whether SBP inhibited the viability of MDA-MB-231 cells, the cells were treated with 0, 10, 20, 30, 40, 50, and 60 μg/ml SBP, and cell viabilities were examined by MTT assay. As shown in Fig. 2a, cell viability was reduced to 82 % with 20 μg/ml SBP and to 42 % with 40 μg/ml SBP. Cell growth was dramatically suppressed by 83 % after treating with 60 μg/ml SBP, when compared to the negative control (0 μg/ml). SBP showed high inhibition of cell population

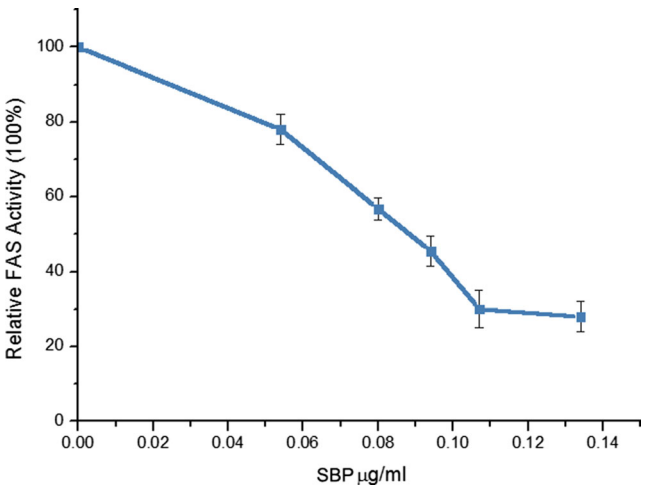

Fig. 1 Inhibitory effects on FAS activities by SBP. The dose-dependent inhibition of FAS by SBP at the concentrations 0–0.14 μg/ml was measured. IC_{50}=0.087±0.008 μg/ml. Values represent the mean±SD of triplicate determinations

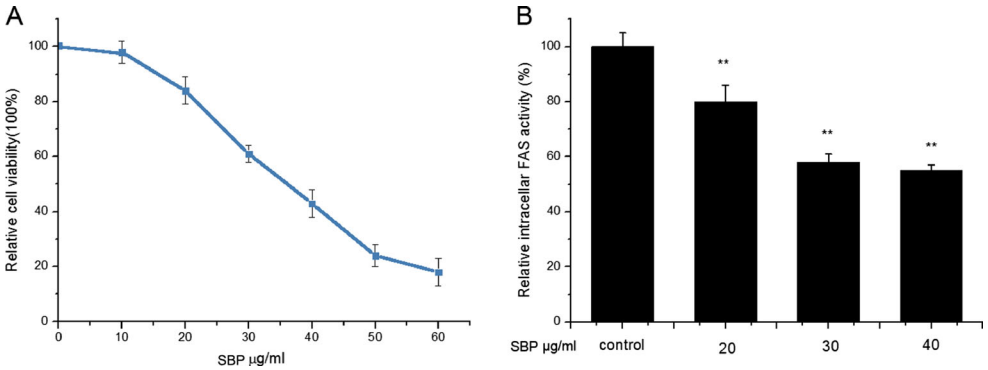

Fig. 2 Dose-dependent inhibitory effects of SBP on cell viability and intracellular FAS activity. **a** Cell viability was determined by MTT assay. MDA-MB-231 cells were incubated with SBP for 24 h at the concentrations 0–60 μg/ml. IC_{50}=37.5±1.0 μg/ml. *Bars* represent means±SD. **b** FAS activity assay was described in the "Materials and methods" section. Data were normalized to control cells without SBP (0 μg/ml). Relative FAS activity was presented as means±SD. *p<0.05 compared to control; **p<0.01 compared to control

growth in a dose-dependent manner with 50 % growth inhibitory concentration (IC_{50}) value of 37.5±1.0 μg/ml.

SBP inhibited FAS activity in MDA-MB-231 cells

Compared with control, SBP significantly inhibited the intracellular FAS activity with a dose-dependent manner. As shown in Fig. 2b, MDA-MB-231 cells were treated with SBP at the concentration of 20, 30, and 40 μg/ml for 24 h. Intracellular FAS activities were reduced to 78.7, 57.3, and 55.1 %, respectively, compared with control.

Apoptotic effect of SBP on MDA-MB-231 cells

After being exposed to 0.1 % DMSO (control) or SBP (20, 40, and 60 μg/ml) for 24 h, apoptosis of MDA-MB-231 cell was demonstrated by Hoechst 33258 and PI dual staining. The Hoechst dye stains the nuclei of all cells and therefore allows to monitor nuclear changes associated with apoptosis, such as chromatin condensation and nuclear fragmentation. PI, on the other hand, is excluded from viable and early apoptotic cells; consequently, PI uptake indicates loss of membrane integrity characteristic of necrotic and late apoptotic cells. In combination with fluorescence microscopy, the selective uptake of the two dyes allows the monitoring of the induction of apoptosis in intact cultures and to distinguish it from non-apoptotic cell death (necrosis). Necrosis is characterized in this system by nuclear PI uptake without chromatin condensation or nuclear fragmentation. Figure 3 showed Hoechst/PI double-stained viable cells, early and late apoptotic cells and necrotic MDA-MB-231 cells, which were exposed to 20, 40, and 60 μg/ml SBP.

To determine whether the reduction of SBP-treated MDA-MB-231 cell viability was due to apoptotic induction, ApoAlert annexin V kit was employed to characterize the death feature of SBP-treated MDA-MB-231 cells. As shown in Fig. 4, a significant population of early (28 %) and late apoptosis (48 %) was observed in after 24-h treatment by SBP (30 μg/ml), demonstrating that SBP results in apoptosis of the MDA-MB-231 cells, consistent with the Hoechst staining result.

Sodium palmitate rescued MDA-MB-231 cells apoptosis induced by SBP

To confirm that the cell apoptosis induced by SBP was related to FAS inhibition, MDA-MB-231 cells were exposed for 24 h to different concentrations of SBP (0, 25, and 50 μg/ml) in the presence of exogenous palmitate (0, 25, and 50 μM), the end

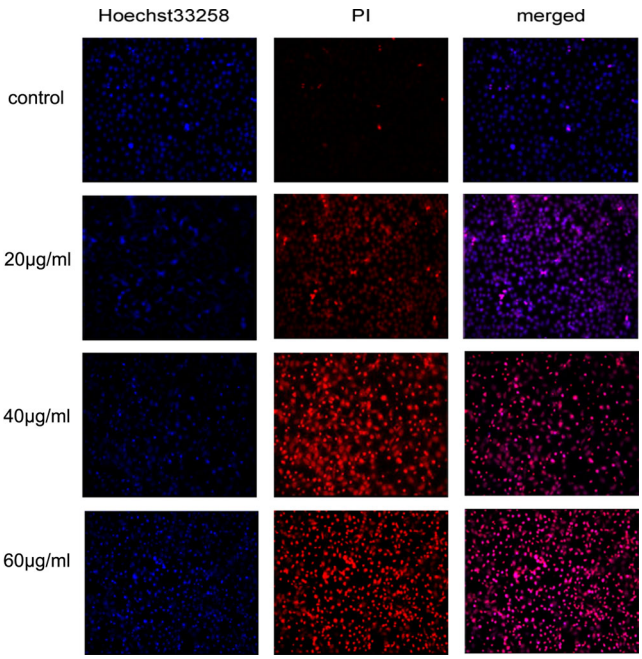

Fig. 3 Hoechst 33258/PI dual staining in SBP-treated MDA-MB-231 cells. Cell culture was performed as described in the "Materials and methods" section. Photos of MDA-MB-231 cells were taken after Hoechst 33258 and PI dual staining; the concentrations of SBP were as follows: 0, 20, 40, and 60 μg/ml. Original magnification, ×200

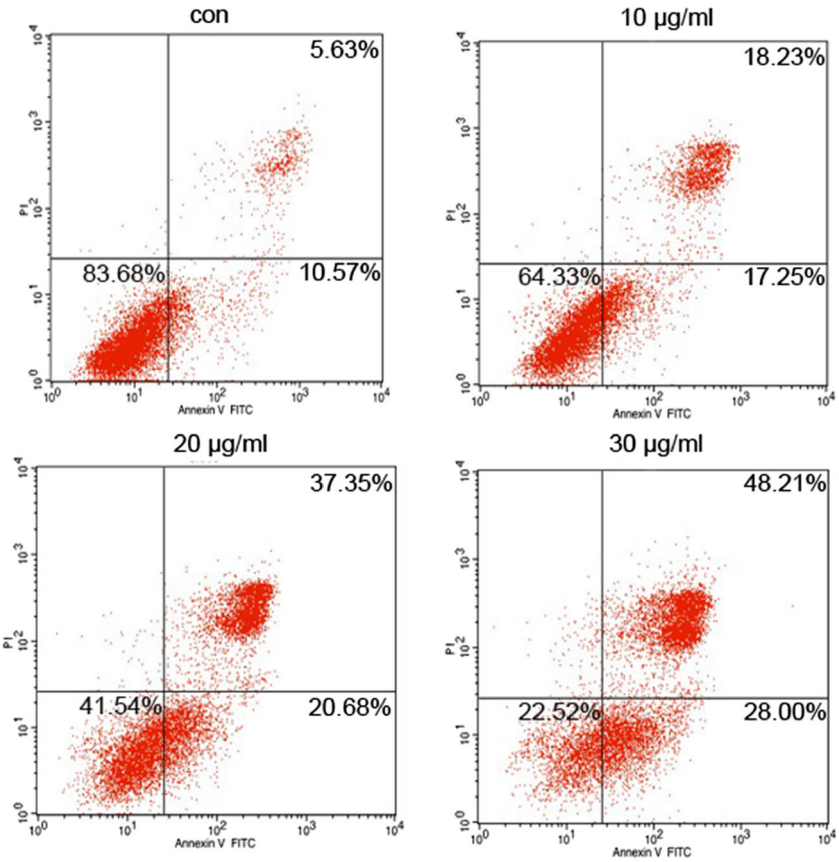

Fig. 4 Flow cytometry analysis of SBP-treated MDA-MB-231 cells. Cells were treated with 0, 10, 20, and 30 μg/ml SBP for 24 h. After annexin V/PI dual staining, the cells were photographed under fluorescence microscope

product of FAS reaction. Palmitate reduced the cytotoxic effects of SBP, as the cell viabilities were significantly increased after the addition of exogenous palmitate. We found that the viability of cells can be restored to some extent and with dose-dependent manner (Fig. 5).

Discussion

Breast cancer is one of the most frequent malignancy occurring in women, especially in the Western Europe and North America. At the same time, interest in the pharmacological effects of functional foods on cancer treatment and prevention has increased dramatically over the past 20 years [34–37]. We have been committed to natural medicine research for human health, and recently, we focus on sea buckthorn, which is a functional food with modern virtues, due to its nutritional and medicinal value. It is primarily valued for its very rich vitamins, flavonoids, lycopene, carotenoids, and phytosterols. Generally it is believed that sea buckthorn is therapeutically important because it is rich with potent antioxidants [38–42]. Although in the recent years, the number of studies investigating the pharmacological activities about sea buckthorn has increased rapidly, there is still limited information available regarding its anticancer activity. The present study was undertaken to assess the anticancer activity of sea buckthorn, using MDA-MB-231 cells. We found that SBP could decrease the viability of MDA-MB-231 cells with the IC_{50} value of 37.5 μg/ml and induce cell apoptosis dose dependently. To our knowledge, this is the first report against cancer cells of SBP.

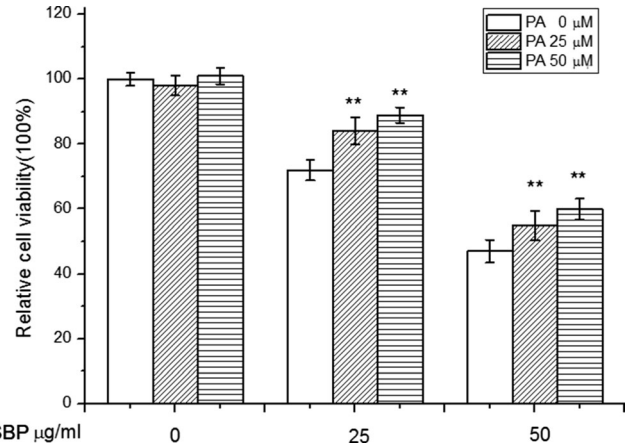

Fig. 5 Sodium palmitate rescued MDA-MB-231 cell apoptosis induced by SBP. Cells were added 0, 25, and 50 μM sodium palmitate in the presence of 0, 25, and 50 μg/ml SBP. After 24 h, MTT assay was used to analyze viable cells. *Bars* represent means±SD. ** $p<0.01$ significantly different from control (0 μM sodium palmitate)

Numerous studies have clearly demonstrated that FAS, a key enzyme participating in lipogenesis and the de novo synthesis of palmitate from Ac-CoA, Mal-CoA, and NADPH, played an important role in converting excess carbon intake into fatty acids in cancer cells [43, 44]. FAS shows low expression in normal tissues, as the requirement of quiescent cells for fatty acids is generally provided via dietary fatty acids. However, in rapidly proliferating cancer cells, such as prostate, ovarian, breast, endometrial, thyroid, and liver carcinomas, FAS always shows overexpression [45, 46]. This suggests that tumors require higher levels of fatty acids than can be acquired from the circulation. Elevated expression of FAS has been linked to poor prognosis and reduced disease-free survival in many cancer types [47]. FAS represents an attractive target for cancer treatment. The present study showed that SBP was a novel FAS inhibitor with an IC_{50} value of 0.087 μg/ml, which was significantly lower than that of cerulenin (20 μg/ml) and EGCG (25 μg/ml), two traditional FAS inhibitors reported before [48, 49].

Based on the evidence that FAS is overexpressed in cancer cells and SBP is a novel FAS inhibitor, we proposed and subsequently set out to demonstrate that SBP induced cancer cell apoptosis via inhibiting intracellular FAS activity.

What we have shown is that SBP produced a dose-dependent decrease in the viability of MDA-MB-231 cells and induced apoptosis certificated by Hoechst 33258, annexin V-FITC, and PI staining. Then, we verified the apoptotic effects of SBP by flow cytometry techniques, and the result is positive, which means that SBP do induce MDA-MB-231 cells apoptosis and, though to a certain extent, SBP might caused some necrosis (Fig. 4). Meanwhile, intracellular FAS activity was inhibited by SBP. These results indicated that SBP indeed affects the synthesis of fatty acids and somehow leads to apoptosis of MDA-MB-231 cells. The biochemical and metabolic bases of FAS overexpression are not well understood. But, the antiproliferative and proapoptotic effects of FAS inhibition have been shown in many cancer cells [24, 50].

The toxicity of palmitate toward a variety of human cell lines has been previously investigated and found that palmitate can induce cell apoptosis dose dependently [51–53]. However, in this study, we observed that when adding palmitate, the final product of FAS catalytic activity, to SBP-treated MDA-MB-231 cells, cell viability was partially rescued. As shown in Fig. 5, with the concentration of exogenous fatty acid raised, the ratio of cell viability was increased. Thereby providing evidence, FAS inhibition is a direct cause in the cytotoxic effects of SBP on cancer cells. These results suggest that cell apoptosis is closely related to fatty acid synthesis.

In conclusion, our study represents the first evaluation of apoptotic activity of SBP on FAS-overexpressed cancer cells. Our present data indicate that SBP can induce MDA-MB-231 cell apoptosis by inhibiting intracellular FAS activity. Although far from complete, our results show that SBP can be considered to have the application potential in treatment of cancer, and they may supply some useful idea and new clues in developing target-directed anticancer drugs for further in vivo studies.

Acknowledgments This work was supported by the Special Science and Technology Projects for Outstanding Young in Life Sciences (KSCX2-EW-Q-19), the "12th five-year plan" period: the basic frontier project in life sciences (KSCX2-EW-J-29), the National Natural Science Foundation of China (81001698), as well as the High-Tech Research and Development Program of Xinjiang (No. 201315108) and the China Postdoctoral Science Foundation (No. 2013M540785).

Conflicts of interest None

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