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SBF-1 preferentially inhibits growth of highly malignant human liposarcoma cells

Wei Chen [a], Xuelong Qian [a], Yue Hu [a], Wei Jin [a], Yunlong Shan [a], Xianying Fang [a], Yang Sun [a], Biao Yu [b], Qiong Luo [a, *], Qiang Xu [a, **]

[a] State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, 163 Xianlin Avenue, Nanjing 210023, China
[b] State Key Laboratory of Bio-organic and Natural Products Chemistry, Shanghai Institute of Organic Academy, Shanghai 200032, China

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ABSTRACT

Frequent local recurrence and metastasis are generally involved in human liposarcoma, but the management is a challenge. There is an urgent need for improved effective therapy. In the present study, we reported that SBF-1, a steroidal glycoside, inhibited the growth of cultured highly malignant human liposarcoma SW872-S cells *in vitro* and *in vivo*. SBF-1 down-regulated the phosphorylation of protein kinase B (AKT) and thus reduced cell adhesion to fibronectin and laminin. Then we found that SBF-1 inhibited the expression of oxysterol binding protein (OSBP) in SW872-S cells, indicating that OSBP may be involved in malignant liposarcoma cell survival. Cancer cell growth and AKT phosphorylation were inhibited significantly upon knockdown of OSBP in SW872-S cells *in vitro*. Taken together, these results suggest that SBF-1 causes an apparent loss of OSBP function in SW872-S cells, resulting in growth inhibition. Based on our findings, OSBP serves as a potential therapeutic target for human liposarcoma.

1. Introduction

Liposarcoma is the most common subtypes of soft tissue sarcoma, originating from adipocytes.[1–3] According to the World Health Organization classification system, liposarcoma can be divided into four types: well-differentiated liposarcoma, dedifferentiated liposarcoma, myxoid/round cell liposarcoma, and pleomorphic liposarcoma.[4–6] Among these four subtypes of liposarcoma, well-differentiated liposarcoma and dedifferentiated liposarcoma account for 40–45%.[7] Treatment options of liposarcoma patients involve surgery, cytotoxic chemotherapy and conventional radiotherapy.[8] For high-grade liposarcoma, frequent local recurrence and metastasis are likely to occur. Moreover, this liposarcoma is highly resistant to chemotherapy and rasiotherapy. All these findings suggest that novel and more effective therapies are needed to meet the needs of liposarcoma paients.

Oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs) constitute a large eukaryotic gene family that transports and regulates the metabolism of sterols and phospholipids.[9] OSBP, the founder member of the ORP family, is identified as a soluble receptor for oxysterols,[10] which are oxidized cholesterol derivatives.[11] OSBP was found to associate with the Golgi complex upon binding of 25OHC or depletion of cellular cholesterol.[12] OSBP/ORPs share a characteristic feature of a conserved C-terminal OSBP-related ligand-binding (ORD) domain.[13] Although the core function is to sense and move lipids, OSBP also plays a role in the regulation of cell signaling pathways.[14] There is growing evidence that OSBP appears to be implicated in cancer development. However, the function is currently unclear.

Our previous studies have demonstrated that SBF-1, a unique saponin glycoside, has a very strong antitumor activity in various cancer types.[15–18] Here we demonstrated for the first time that OSBP played an important role in liposaroma development. Small molecule compound SBF-1 decreased OSBP expression to show its antitumor activity against highly malignant liposarcoma *in vitro* and *in vivo*. These results may offer a novel approach to treat advanced liposarcoma.

* Corresponding author. Fax: +86 25 89687620.
** Corresponding author.
E-mail addresses: qiongluo@nju.edu.cn (Q. Luo), molpharm@163.com (Q. Xu).
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2. Materials and methods

2.1. Mice

Specific pathogen-free, six-week-old female NCR-nu/nu (nude) mice were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.2. Cell culture

Human liposarcoma cell line SW872 cells were purchased from the American Type Culture Collection (Rockville, MD). SW872-S cells with high tumorigenicity were established by *in vivo* re-inoculation approach as described previously.[19] Cells were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies), 100 U/mL penicillin, 100 mg/ml streptomycin under a humidified 5% (v/v) CO_2 atmosphere at 37 °C.

2.3. Reagents

SBF-1 is a synthetic steroidal glycoside [14]. For *in vitro* experiments, SBF-1 was dissolved in DMSO to a concentration of 20 mM (stock solution); for *in vivo* assay, SBF-1 was dissolved in DMSO to a concentration of 10 mg/ml (stock solution), and stored at −20 °C. The final DMSO concentration did not exceed 0.1% throughout the study. 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sunshine Biotechnology (Nanjing, China). Fibronectin and laminin were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies against phospho-AKT, AKT, LC3, cleaved Caspase 9, XBP1 and phosphor-PERK were purchased from Cell Signal Technology (Beverly, MA). Antibody against OSBP, GAPDH and integrin α6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.4. MTT assay

The cells were seeded into 96-well plates, and incubated with various concentrations of SBF-1 at 37 °C for indicated time periods. Four hours before measurement, 20 μl per well MTT solutions (4 mg/ml in PBS) were added into the 96-well plates. After incubation at 37 °C for 4 h, the plates were measured under an FL × 800 Fluorescence Microplate Reader (BioTek, Winooski, VT, USA) at 570 nm. The IC50 value was defined as the concentrations that cause a 50% loss of cell viability.

2.5. Flow cytometry

SW872 and SW872-S cells treated with different concentrations of SBF-1 for indicated time periods were incubated with respective antibodies for 30 min. Isotype matched mouse IgG served as negative control. Cells were then washed twice with icecold PBS. Integrin expression was analyzed by a FACSCalibur Flow Cytometry and CellQuest software (BD Biosciences).

2.6. Western blot

Proteins were extracted in lysis buffer (30 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (Cell Signaling Technology).

2.7. Cell adhesion assay

Cell adhesion assay was performed using methods published before[20] with some modifications. In brief, 96-well flat-bottom plates were coated with 50 μl fibronectin (10 μg/ml) or laminin (10 μg/ml) in PBS overnight at 4 °C and then blocked with 0.2% bovine serum albumin (BSA) for 2 h at room temperature followed by washing three times. Cells were added into each well in triplicate and incubated for 30 min at 37 °C. Plates were then washed three times with PBS to remove unbound cells. Numbers of cells remained bound to the plates were analyzed by MTT assay. After subtraction of the background cell binding to BSA-coated wells, the percentage of adherent cells was calculated by dividing the optical density of the adherent cells by that of the initial input cells.

2.8. Transient transfection in cells

Transient transfections were performed to transfer vectors into cells. Briefly, 2 μl lipofectamine 2000 (Invitrogen) and 2 μg plasmids were diluted in 50 ml opti-MEM (GIBCO) and mixed softly, then incubated at room temperature for 20 min before addition of another 800 ml opti-MEM. At last, the mixtures were spread onto cells and the cells were incubated at 37 °C for 6 h before switched to DMEM supplemented with 10% FBS. Then the cells were incubated at 37 °C for another 18 h. In the end, the cells were collected and prepared for the following experiments.

2.9. In vivo tumor xenografts model

2×10^6 SW872-S cells (in 0.1 ml PBS) were injected subcutaneously into the right flanks of nude mice. Four days later, all the mice formed visible tumors. Then, the mice were distributed into four groups (n = 6) according to tumor volumes. 10 μg/kg and 30 μg/kg SBF-1 were intraperitoneally injected to the tumor-bearing mice every three days. 15 mg/kg taxol was intraperitoneally injected to the tumor-bearing mice once every week. Body weight and tumor volumes were measured and recorded every three days. Long diameter (L) and short diameter (S) of a tumor were measured with a vernier caliper and the tumor volume was calculated as follows: $L \times S^2/2$.

2.10. Statistical analysis

Data are expressed as means ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Student's two-tailed t-test. $P < 0.05$ was considered significant.

3. Results

3.1. SBF-1 strongly inhibits the proliferation of malignantly progressed liposarcoma subpopulation of SW872 cells

A new sub-cell line derived from human liposarcoma cells SW872 (SW872-S) was characterized with much stronger tumor-initiating potential in mice. As shown in Fig. 1B, SW872-S cells grew faster than SW872 cells *in vitro*. Then SW872 cells and

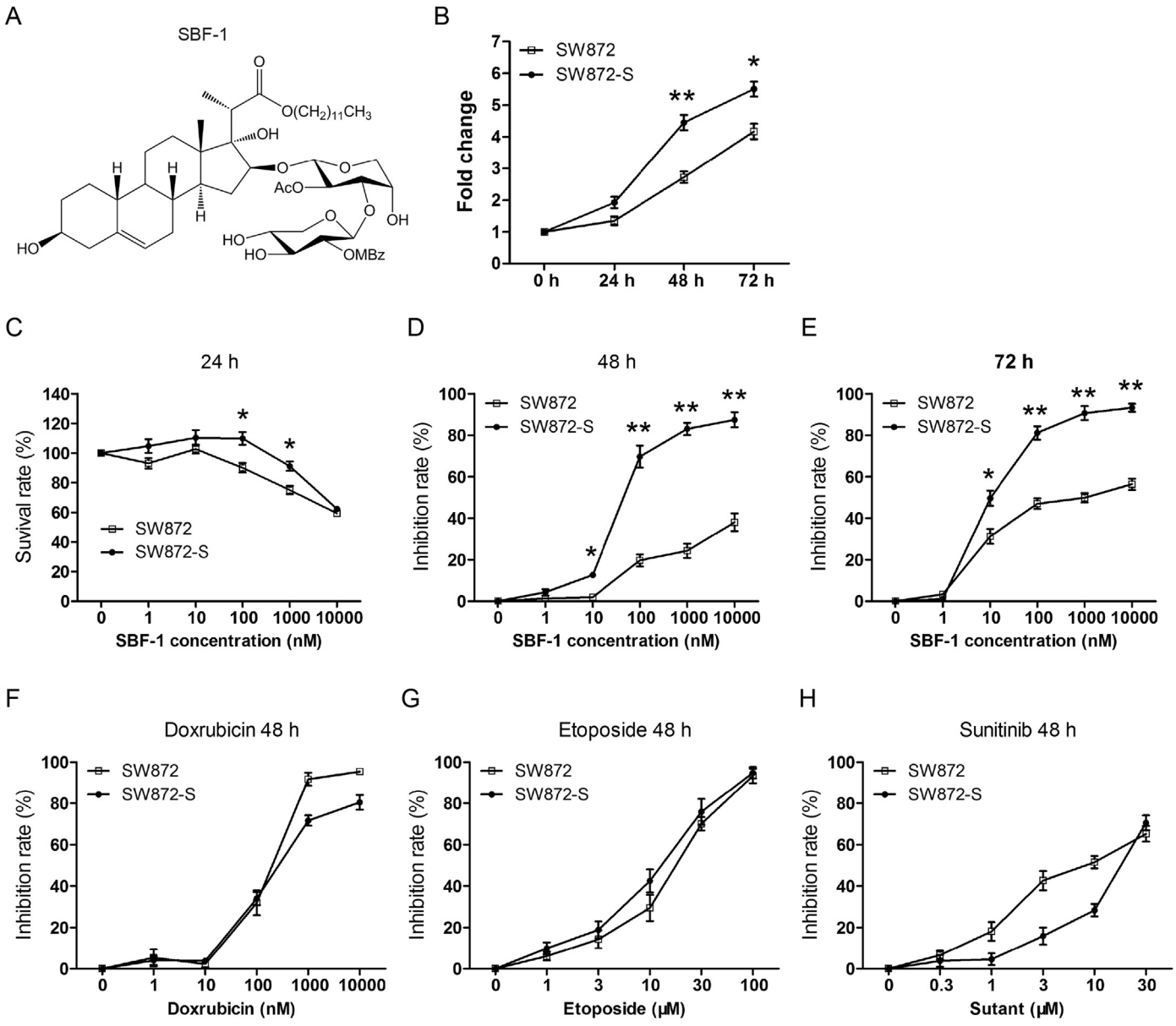

Fig. 1. SBF-1 strongly inhibited the proliferation of SW872-S cells. (A) The chemical structure of SBF-1. (B) SW872 and SW872-S cells were seeded in 96-well plates. The cell proliferation rate was determined by MTT assay at 0 h, 24 h, 48 h and 72 h after the cells adhere to the plate. (C–E) SW872 and SW872-S cells were seeded in 96-well plates and incubated with various concentrations of SBF-1 for 24 h, 48 h and 72 h. The inhibitory rate of SBF-1 on the cell proliferation was determined by MTT assay. Data are mean ± SEM of three independent experiments, and each experiment includes triplicate sets. (F–H) SW872 and SW872-S cells were seeded in 96-well plates and incubated with doxorubicin, etoposide or sunitinib for 48 h. The inhibitory rate of SBF-1 on the cell proliferation was determined by MTT assay. Data are mean ± SEM of three independent experiments, and each experiment includes triplicate sets.

SW872-S cells were incubated with different concentrations of SBF-1 for indicated time and we tested the cytotoxic effect of SBF-1. SBF-1 concentration- and time-dependently inhibited growth of SW872 cells and SW872-S cells (Fig. 1C–E). To our surprise, the highly malignant SW872-S cells exhibited more sensitivity to SBF-1 than SW872 cells (IC50 was 26.03 nM for SW872-S and 995.4 nM for SW872, respectively). By contrast, SW872-S cells were not sensitive to etoposide and significantly more resistant to doxorubicin and sunitinib than SW872 cells (Fig. 1F–H).

3.2. SBF-1 induces apoptosis in SW872-S cells

We next analyzed the effect of SBF-1 on apoptosis in SW872 cells and SW872-S cells. Cells were seeded into 6-well plates and incubated with different concentrations (0, 100, 1000 nM) of SBF-1 for 24 h. As shown in Fig. 2A, Flow cytometry analysis showed that SBF-1 induced apoptosis both in SW872 cells and SW872-S cells. Notably, SBF-1 only showed a slight induction of apoptosis on SW872 cells. While SBF-1-treated SW872-S cells showed much increased apoptotic cell counts in a dose-dependent manner. To further determine the potential mechanism of SBF-1-induced apoptosis, we detected the expressions of several apoptosis related proteins by western blotting. After incubating with 100 nM SBF-1 for 0, 1.5, 3, 6, 12, and 24 h, the protein levels of spliced form of X-box-binding protein 1 (XBP1s), cleaved caspase-9 and spliced form of LC3 were significantly increased (Fig. 2B). These results suggest that the growth inhibition induced by SBF-1 is associated with cell apoptosis. Notably, the apoptotic signaling was enhanced in SBF-1-treated SW872-S cells (Fig. 2C).

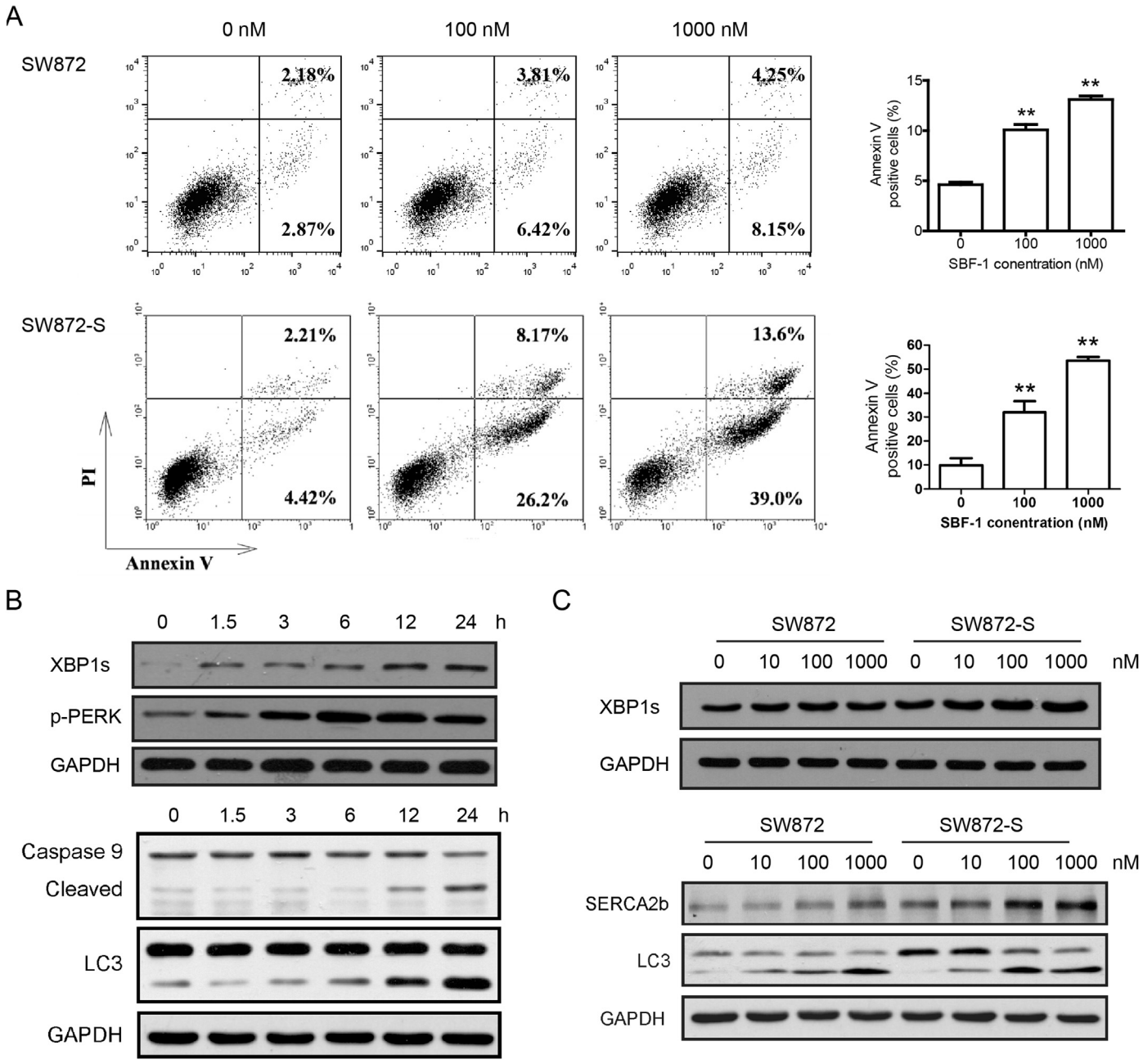

Fig. 2. SBF-1 induced apoptosis of SW872-S cells. (A) SW872 and SW872-S cells were seeded in 6-well plate and incubated with 0, 100 and 1000 nM SBF-1 for 24 h. The apoptosis of SW872 and SW872-S cells was determined by Annexin V/PI staining. Annexin V positive SW872 and SW872-S cells were shown in bar graph. Data are mean ± SEM of three independent experiments. **P < 0.01. (B) SW872-S cells were treated by 100 nM SBF-1 for the indicated time. The protein levels of XBP1s, p-PERK, Caspase 9 and LC3 were determined by Western blotting. Data are representative of three independent experiments. (C) SW872 and SW872-S cells were incubated with 0, 10, 100 and 1000 nM SBF-1 for 24 h. The protein levels of XBP1s, SERCA2b and LC3 were determined by Western blotting. Data are representative of three independent experiments.

3.3. SBF-1 down-regulates the phosphorylation of AKT and inhibits adhesive ability of SW872-S cells

As we known, AKT is one of the most important intracellular pathways which promote proliferation of tumor cells.[21,22] In the following study, the phosphorylation of AKT at Ser473 was examined by western blotting. We found that p-AKT was significantly down-regulated in a concentration-dependent manner (Fig. 3A). This inhibition was greatly augmented in SBF-1-treated SW872-S cells. In addition, AKT is crucial for adhesion of tumor cells and inhibition of AKT activity leads to decreased metastasis.[23] From above results, we know that SBF-1 efficiently inhibited the phosphorylation of AKT. We supposed that SBF-1 could inhibit adhesion of SW872-S cells. Cells were treated with different concentrations (0, 10, 30, 100, and 300 nM) of SBF-1 for 24 h, and then adhesive ability to fibronectin and laminin were detected. As shown in Fig. 3B, SBF-1 showed a remarkable inhibition on adhesive ability of SW872-S cells to fibronectin and laminin. SW872-S cells show elevated expression of integrin α6,[19] so then we analyzed the effect of SBF-1 on integrin α6 expression. Fluorescence-activated cell sorting (FACS) analyzing and western blotting analysis revealed that SBF-1 inhibited the expression of integrin α6 on SW872-S cell

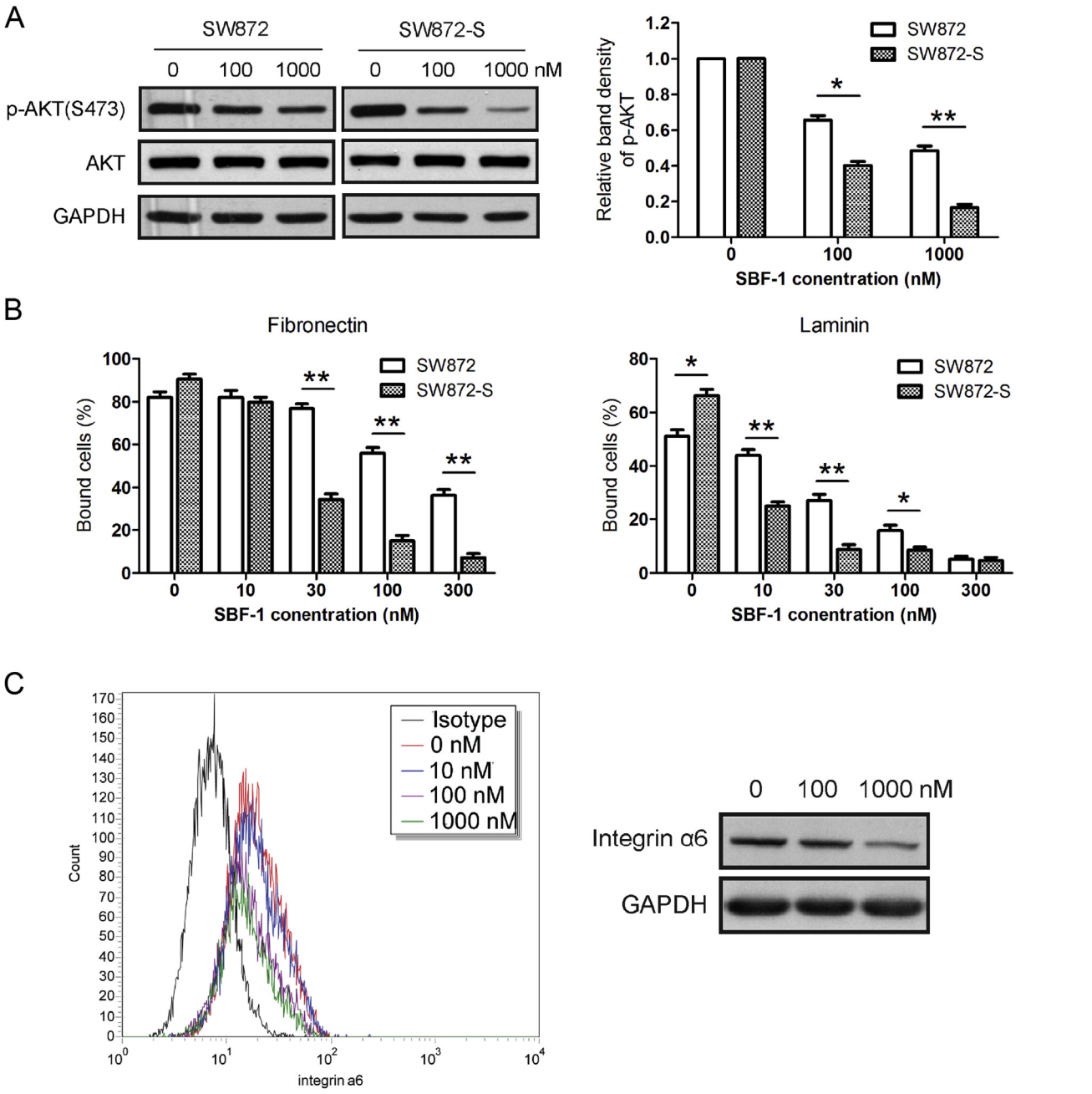

Fig. 3. SBF-1 inhibited the phosphorylation of AKT and adhesive ability of SW872-S cells. (A) SW872 and SW872-S cells were seeded in 6-well plate and incubated with 0, 100 and 1000 nM SBF-1 for 24 h. The phophorylation of AKT was detected by Western blotting. (B) SW872 and SW872-S cells were seeded in 60 mm dish, and treated by various concentrations of SBF-1. Twenty-four hours later, cells were harvested for adhesion assay. Data are mean ± SEM of three independent experiments. (C) SW872-S cells were seeded in 6-well plate and incubated with SBF-1 of 0, 10, 100 and 1000 nM. Twenty-four hours later, cells were harvested and stained with anti-CD49f antibody. The expression of integrin α6 was detected by both flow cytometry and Western blotting.

surface (Fig. 3C). These results suggest that SBF-1 suppressed integrin α6 expression and thus inhibited adhesive ability of SW872-S cells, which may due to the inhibition of AKT phosphorylation.

3.4. SBF-1 shows stronger suppression on the expression of OSBP in SW872-S cells

Previous studies have showed that OSW-1 targets oxysterol binding protein (OSBP) and its closest paralog, OSBP-related protein 4L (ORP4L) to inhibit the growth of cultured human cancer cell lines.[24] As an analog of OSW-1, we supposed that SBF-1 might affect the expression of OSBP. As expected, we found that SBF-1 decreased the protein level of OSBP in a dose-dependent manner both in SW872 cells and SW872-S cells, which was more effective in SW872-S cells (Fig. 4A). Real-time PCR analysis demonstrated that SBF-1 did not affect OSBP gene expression in SW872-S cells (Fig. 4B). To determine if SBF-1 induced proteasome-dependent degradation of OSBP, we administered proteasome inhibitor MG-132 with SBF-1. MG-132 alone did not affect OSBP expression, but it completely blocked the OSBP degradation induced by SBF-1 in SW872 cells and SW872-S cells (Fig. 4C). To further investigate the

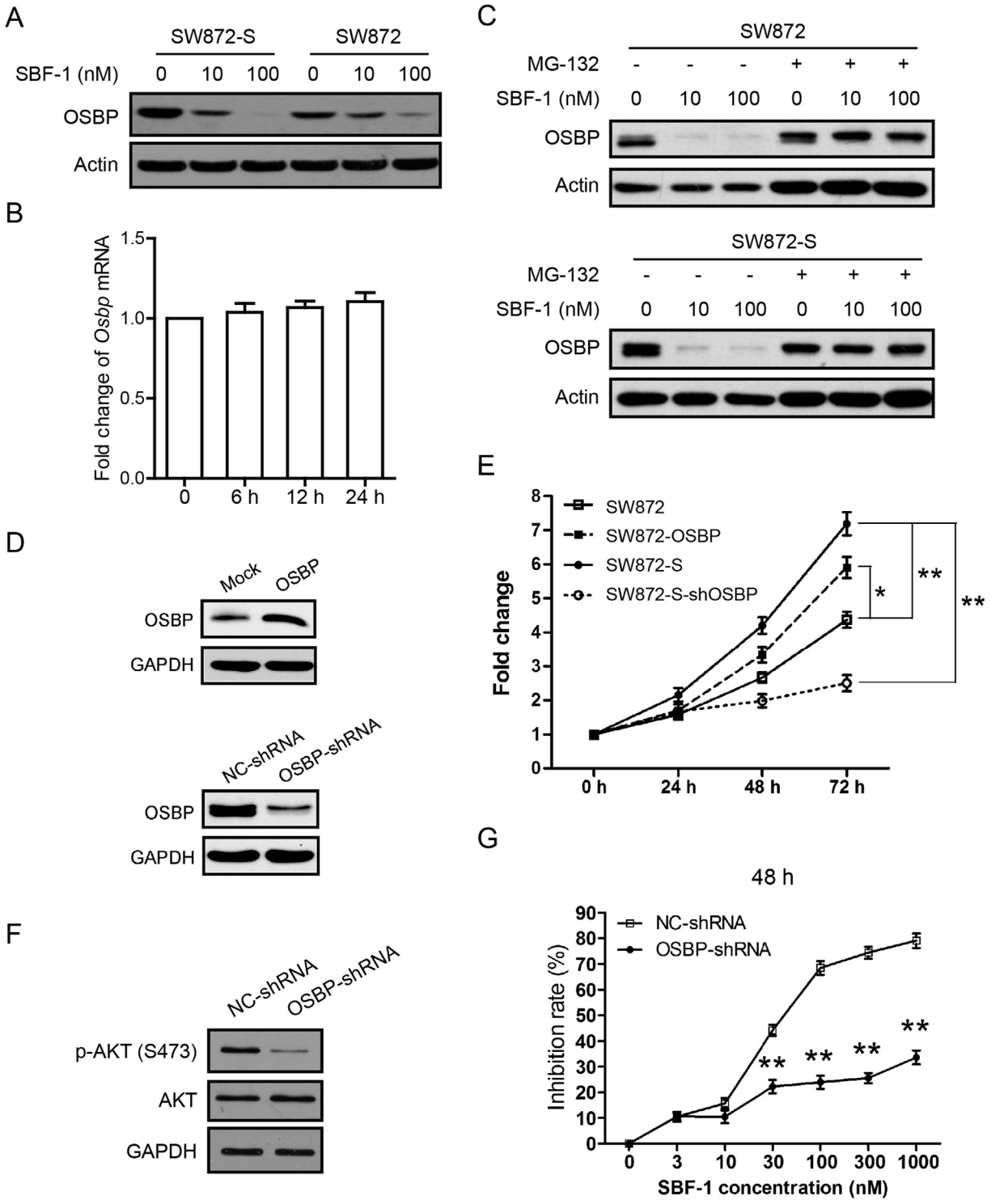

Fig. 4. SBF-1 decreased OSBP expression in SW872-S cells. (A) SW872 and SW872-S cells were seeded in 6-well plate and incubated with 0, 10 and 100 nM SBF-1 for 24 h. The protein levels of OSBP were determined by Western blotting. (B) SW872-S cells were seeded in 6-well plate and incubated with 100 nM SBF-1 for 6 h, 12 h and 24 h. The mRNA levels of OSBP were determined by real-time PCR. (C) Immunoblot analysis of OSBP in cell lysates form SW872 and SW872-S cells treated with MG132 for 1 h and then treated with different doses of SBF-1 for 24 h. (D) SW872 cells were overexpressed with pcDNA3.1(−) or pcDNA3.1(−)–OSBP vectors for 24 h and SW872-S cells were interfered with scramble-shRNA or OSBP-shRNA for 48 h. Then the protein levels of OSBP were determined by Western blotting. (E) Each kind of cells was seeded in 96-well plates with 3000 cells per well and the cell proliferation curve was determined by MTT assay at 0 h, 24 h, 48 h and 72 h. Data are mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01. (F) SW872-S cells were interfered with scramble-shRNA or OSBP-shRNA for 48 h. The phophorylation of AKT was detected by Western blotting. (G) SW872-S cells were interfered with scramble-shRNA or OSBP-shRNA for 48 h. Then cells were seeded in 96-well plates and incubated with various concentrations of SBF-1 for 48 h. The inhibitory rate of SBF-1 on the cell proliferation was determined by MTT assay. Data are mean ± SEM of three independent experiments, and each experiment includes triplicate sets. *P < 0.05, **P < 0.01.

role of OSBP in the antitumor effects of SBF-1, we overexpressed OSBP in SW872 cells and knocked down OSBP in SW872-S cells (Fig. 4D). As shown in Fig. 4E, OSBP overexpression promoted the growth of SW872 cells, while OSBP silence greatly suppressed the growth of SW872-S cells. Consistent with SBF-1 treatment, the knockdown of OSBP inhibited the phosphorylation of AKT in SW872-S cells (Fig. 4F). In addition, OSBP shRNA knockdown desensitized cells to SBF-1 in SW872-S cells (Fig. 4G), indicating that OSBP was required for the suppression of SW872-S cell growth by SBF-1.

3.5. SBF-1 inhibits tumor growth at a very low dose in SW872-S xenografts with decreased OSBP levels

To evaluate the antitumor effect of SBF-1 *in vivo*, we subcutaneously injected 2×10^6 SW872-S cells into the right flank of nude mice. Four days after inoculation, when all mice formed tumors, we distributed the mice into four groups. Vehicle (0.1% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS)) and indicated doses of SBF-1 were administrated intraperitoneally to the mice every three days, while taxol (positive control) was administrated intraperitoneally once one week. At the same time, we measured and recorded tumor volumes and body weights every three days. SBF-1 at the very low dose of 30 μg/kg significantly inhibited the growth of tumor xenografts (Fig. 5A). It should be noted that at above doses SBF-1 did not decrease the body weights of mice while taxol did (Fig. 5B). We then detected the expression of OSBP in tumor xenografts. As expected, SBF-1 decreased OSBP protein level (Fig. 5C).

4. Discussion

Liposarcoma is the most common soft tissue sarcomas, and the effective therapeutic approach is scarce because of high risk of recurrence and chemoresistance. This prompts us to develop new targets for the treatment of liposarcoma.

In this study, we found that low concentrations of SBF-1 significantly inhibited the growth of SW872-S cells, a subgroup of SW872 cells with high tumorigenicity in nude mice (Fig. 1B–D). Then SBF-1 was found to induce apoptosis in SW872-S cells in a concentration-dependent manner. Increased levels of unfolded/misfolded proteins can disturb ER homeostasis, and thus trigger the unfolded protein response (UPR).[25,26] When UPR signaling is prolonged or excessive, cellular dysfunction and cell death often occur. We found that SBF-1 increased XBP1s protein level, promoted PERK phosphorylation and caspase-9 cleavage, suggesting that SBF-1 activated ER stress and thus enhanced apoptosis of SW872-S cells.

PI3K/AKT pathway is one of the most frequently altered intracellular signaling pathways in human cancers and has been reported to be a key regulator in survival and growth of tumors.[27] Herein we showed that the phosphorylation of AKT at serine 473 was remarkably reduced by SBF-1 treatment. In addition, AKT also mediates the process of tumor metastasis and cell adhesion.[28] In the following study, we observed that SBF-1 significantly inhibited adhesion of SW982-S cells to fibronectin and laminin. It was reported that SW872-S cells revealed higher adhesive ability than SW872 cells due to upregulated expression of integrin α6 in SW872-S cells. FACS and western blot assay showed that integrin α6 was down-regulated by SBF-1.

To investigate how SBF-1 exhibited its strong antitumor activity in highly malignant human liposarcoma, the expression of OSBP was tested in SW872 cells and SW872-S cells. Our results clearly showed that SBF-1 suppressed OSBP protein expression both in SW872 cells and SW872-S cells. These results strongly suggest that OSBP may play a role in cancer cell survival. In previous study, OSBP has been reported to function as a sterol sensing scaffolding protein which is implicated in vesicle transport, lipid metabolism, and signal transduction.[29] Here we found that depletion of OSBP caused cell growth inhibition, while OSBP overexpression promoted the growth of SW872 cells. AKT is one of the most important molecules in cellular processes and its activation has been described in human liposarcomas.[30] AKT exerts its antiapoptotic and prosurvival effects through phosphorylating and blocking some pro-apoptotic proteins and influencing some transcription factors which may induce anti-apoptotic genes.[31] Several studies reported that AKT/PI3K inhibitors induced apoptosis and reduced viability of liposarcomas cells.[32,33] However, AKT inhibitor could not reduce OSBP levels (data not shown). On the other hand, the knockdown of OSBP suppressed cell growth (Fig. 4C) and inhibited the phosphorylation of AKT in SW872-S cells (Fig. 4D). These results suggest that the major effect of SBF-1 is on OSBP degradation and then inhibition of AKT phosphorylation. Combined with these data, we supposed that SBF-1 might induce OSBP degradation and caused an apparent loss of OSBP function in SW872-S cells, resulting in growth inhibition,

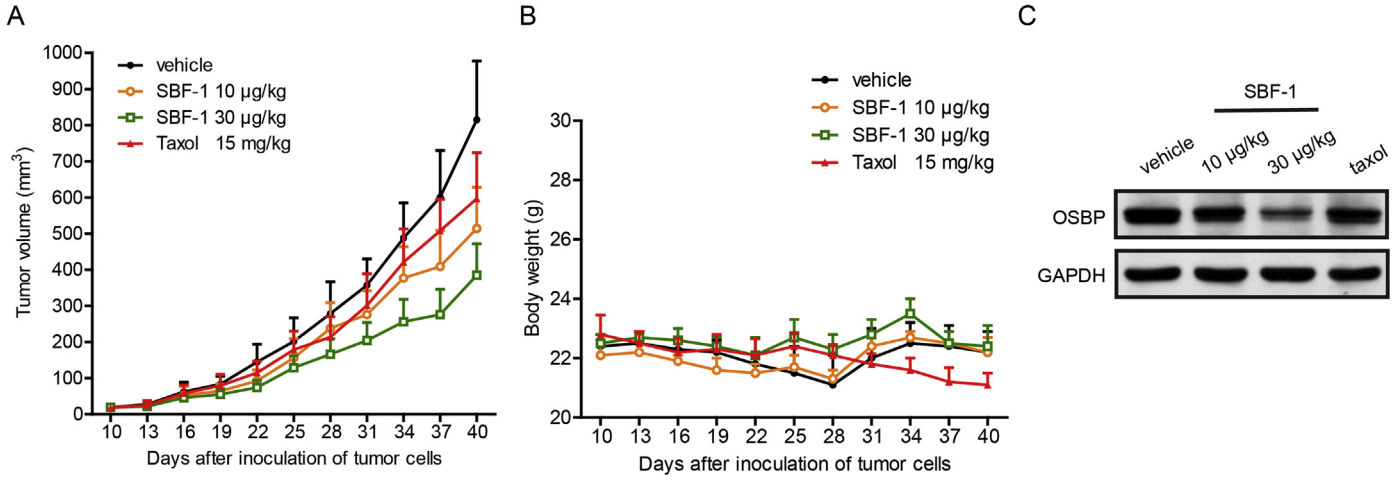

Fig. 5. SBF-1 inhibited tumor growth at a very low dose in SW872-S xenografts with decreased OSBP levels. (A–B) SW872-S cells (2×10^6 cells in 100 μl PBS) were injected subcutaneously into the right flank of nude mice. Four days after innoculation (100% mice formed tumors), the mice were distributed into four groups according to the tumor size (n = 6). Then the mice were administrated with the indicated concentrations of SBF-1 every three days, or taxol every week by intraperitoneal injection. The tumor size and body weight were recorded every three days. (C) The protein levels of OSBP in tumor tissues were determined by Western blotting.

which might explain the strong antitumor effect of SBF-1. Furthermore, *in vivo* studies indicated that 15 and 30 μg/kg SBF-1 significantly inhibited growth of lipodarcoma xenografts, with decreased OSBP expression. Furthermore, we found that the protein expression of OSBP was higher in SW872-S cells than in SW872 cells. Considering that OSBP silence greatly suppressed the growth of SW872-S cells, it is likely that OSBP may play a more important role in the growth of SW872-S cells than that of SW872 cells, which finally lead to enhanced SBF-1 sensitivity in SW872-S cells.

Taken together, our discovery suggests that OSBP could be a therapeutic target for highly malignant human liposarcoma and SBF-1 might be a novel OSBP inhibitor to induce cell growth suppression.

Conflict of interest

The authors have no conflict of interest to declare.

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