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Original Article

Role and mechanism of Sophoridine on proliferation inhibition in human glioma U87MG cell line

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Abstract: Sophoridine, a natural product obtained from medicinal plants, which has a variety of pharmacological effects, including anti-cancer effects, and selectively induces apoptotic cell death in a variety of human cancer cells in vitro and in vivo; however, its mechanism of action needs to be further elaborated. In this study, we investigated the effects of Sophoridine on the induction of apoptosis in human Glioma U87MG cells. Here, we found that Sophoridine can significantly inhibited cell proliferation, G2/M phase arrest, induced cell apoptosis and caused reactive oxygen species (ROS) generation and GSH content reduction. Sophoridine also triggered significant down-regulated the expression of p27, CDK2, Survivin, Livin, Bcl-2, E2F1 and the transcriptional activity of FoxM1, NF-κb and AP-1, meanwhile, up-regulated the expression of caspase-3/8, p53, Smac, c-JNK and p38-MAPK. Moreover, we found that Sophoridine significantly inhibited ubiquitin-proteasome in tumor cells. In conclusion, Sophoridine shows obvious anti-cancer activity on glioma cells by inducing cell apoptosis, inducing ROS accumulation, and activating mitochondrial signal pathways. Eventually, we believe Sophoridine could be used as a new drug for the treatment of glioma.

Keywords: Glioma, U87MG, cell apoptosis, Sophoridine

Introduction

Glioma is the most common malignancy. In the 2014 World Cancer Report issued by the WHO, glioma still ranks the first in the global cancer prevalence and mortality. In the past several decades, considerable advances in glioma treatment have taken place, including surgical operation, radiotherapy, chemotherapy and targeted therapy [1]. Even so, the prognosis of glioma remains poor; as shown by the World Cancer Report, glioma is still the most common cancer with the highest mortality. Therefore, it is essential to search for better treatments and potential preventive measures.

Sophoridine is a monomeric alkaloid extracted from sophora alopecuroides L. of leguminosae sophora and found to have anti-tumor effect. Using a cell-based small-molecule screening method in this study, the authors discovered that Sophoridine was able to selectively kill tumor cell without harming the normal cells, attributable to the effect of Sophoridine to increase intracellular ROS level and enhance apoptosis [2-3]. Further study showed that Sophoridine suppressed tumor by inhibiting the activity of ubiquitin-proteasome [4]. Therefore, Sophoridine is a natural anti-tumor product potentially having high practical values. However, studies examining the effects of Sophoridine on glioma are generally limited. Accordingly, this study not only mainly investigated the inhibitory effect of Sophoridine on human glioma U87MG but also carried out in-depth research on the oxidative stress and the inhibition of ubiquitin-proteasome.

Materials and methods

Cell culture

U87MG was cultured in 10 cm culture dishes in a 37°C incubator containing 5% CO_2 and saturated humidity. The culture medium was 90% F-12K and 10% FBS. The 0.25% trypsin-EDTA was used for digestion and passaging. Cells of logarithmic growth phase were used in all experiments.

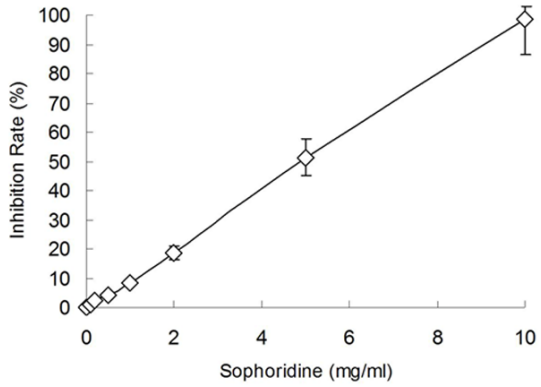

Figure 1. The inhibitory effect of Sophoridine on U87MG cell proliferation. U87MG cells were treated with Sophoridine at different concentrations (0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/mL) for 24 h. Proliferation of tumor cells was measured using the MTT method, and the inhibition rate (%) was calculated.

Detection of Sophoridine's inhibitory effect on tumor cells with MTS assay

The cells of logarithmic growth phase were seeded in 96-well microplate at 3~4 × 10^4 cells/mL, 100 μL/well and cultured overnight to allow cell adhesion. Then, 0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/mL of Sophoridine were added to the corresponding test wells to continue the culture for 24 h. After the culture medium was removed, MTS reagent was added in accordance with the kit instructions. Finally, the OD value was measured at 490 nm wavelength using a microplate reader to calculate the inhibition rate of the drug on the cells. Inhibition rate = (1-OD of experimental group/OD of control group) × 100%. The fitting inhibition curve was plotted using the logarithmic concentration of Sophoridine as abscissa and the inhibition rate as ordinate. The compound concentration corresponding to 50% inhibition rate was the IC_{50}.

Detection of apoptosis and cell cycle with flow cytometry

The tumor cells of logarithmic growth phase were seeded in 6-well plates to continue the culture for 24 h until the cell fusion was preferably 70%~80%. PBS or 1 mg/ml and 2 mg/ml of Sophoridine was added to the test wells to continue the culture for 24 h. Then, the cells were harvested after digestion with trypsin and stained with PI/Annexin V-FITC; the apoptotic cells were detected with a flow cytometer. To detect the effects of Sophoridine on tumor cell cycle, PBS or 0.5 mg/ml and 1 mg/ml of Sophoridine was added to the test wells to continue the culture for 24 h. Then, the cells were harvested after digestion with trypsin and stained with PI; the cell cycle distribution was detected with a flow cytometer.

Determination of intracellular ROS and GSH contents with biochemical assay

U87MG were seeded in 6-well plates and cultured overnight to allow cell adhesion. Then, 0.5 mg/ml and 1 mg/ml of Sophoridine was added using PBS as the control to continue the culture for 24 h. ROS was detected with fluorescent probe DCFH-DA; this probe is able to enter the living cells freely and hydrolyzed to produce DCFH under the action of intracellular esterase, while the latter cannot pass through the cell membrane and can generate detectable fluorescent DCF under the action of ROS. In addition, the cell lysis buffer for Western blotting was used to lyse the cells for GSH detection that was accomplished by its reaction with chromogenic substrate DTNB [5,5'-dithiobis-(2-nitr-ben-zoic acid)] to produce yellow TNB and GSSG. All the specific procedures followed the kit instructions.

Detection of the changes in tumor-related gene mRNA expression with Real-time PCR

U87MG were seeded in 6-well plates and cultured overnight to allow cell adhesion. Then, 0.5 mg/ml and 1 mg/ml of Sophoridine was added using PBS as the control to continue the culture for 24 h. Cells were harvest and extract the total mRNA. The expression of mRNA was detected by Real-time PCR assay. The PCR reactive condition was as following: decontamination at 65°C for 60 s, denaturation at 95°C for 30 s, and at 65°C for 40 s by 40 cycles. β-actin was used as an internal reference. The oligonucleotide sequences were shown: p27, Forward: 3'-AGGACACGCATTTGGTGGA-5', Reverse: 3'-TAGAAGAATCGTCGGTTGCAGGT-5'; CDK2, Forward: 3'-CTCCTGGGCTCGAAATATTATTCCACAG-5', Reverse: 3'-CCGGAAGAGCTGGTCAATCTCAGA-5'; Survivin, Forward: 3'-GCATGGGTGCCCCGACGTTG-5', Reverse: 3'-GCTCCGGCCAGAGGCCTCAA-5'; Livin, Forward: 3'-CTGGTCAGAGCCAGTGTTCCT-5', Reverse: 3'-TCATAGAAGGAGGCCAGACG-5'; Bcl-2, Forward: 3'-ACGGG-

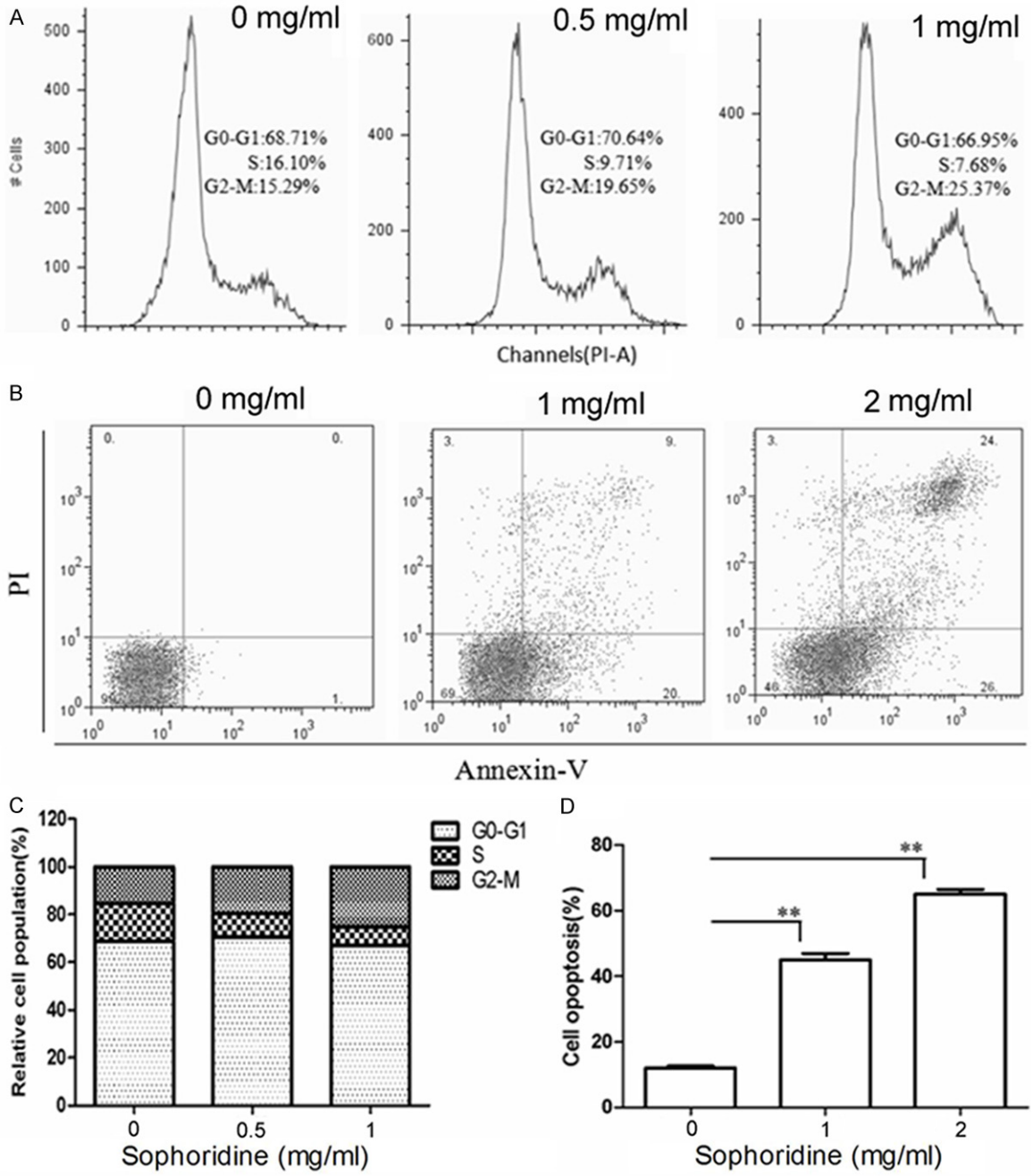

Figure 2. Sophoridine induced cell-cycle arrest and apoptosis in U87MG cells. A: Cells were treated with 0.5 mg/mL and 1 mg/mL Sophoridine for 24 h. PI staining was used to analyze the cell cycle distribution; B: 1 mg/mL and 2 mg/mL Sophoridine exposure in U87MG cells as assessed by Annexin V-fluorescein isothiocyanate and propidium iodide (PI) double staining and fluorescence-activated cell sorter analysis; C: Histogram of cell cycle distribution of U87MG cells. D: Histogram of the percentage of apoptotic cells in U87MG cells. Bars mean ± SD. n = 3. The statistical significance was considered as *P < 0.05 and **P < 0.01 where compared with control.

GTGAACTGGGGGAGGA-5', Reverse: 3'-TGTTTGGGGCAGGCATGTTGACTT-5'; E2F1, Forward: 3'-ACTCCTCGCAGATCGTCATCATCT-5', Reverse: 3'-GGACGTTGGTGATGTCATAGATGCG-5'; p53, Forward: 3'-AACCTACCAGGGCAGCTACG-5', Reverse: 3'-TTCCTCTGTGCGCCGGTCTC-5'; Smac, Forward: 3'-AGCTGGAAACCACTTGGATGA-5', Reverse: 3'-GAATGTGATTCCTGGCGGTTA-5'; β-actin, Forward: 3'-TGAGCGCGGCTACAGCTT-5', Reverse: 3'-TCCTTAATGTCACGCACGATTT-5'.

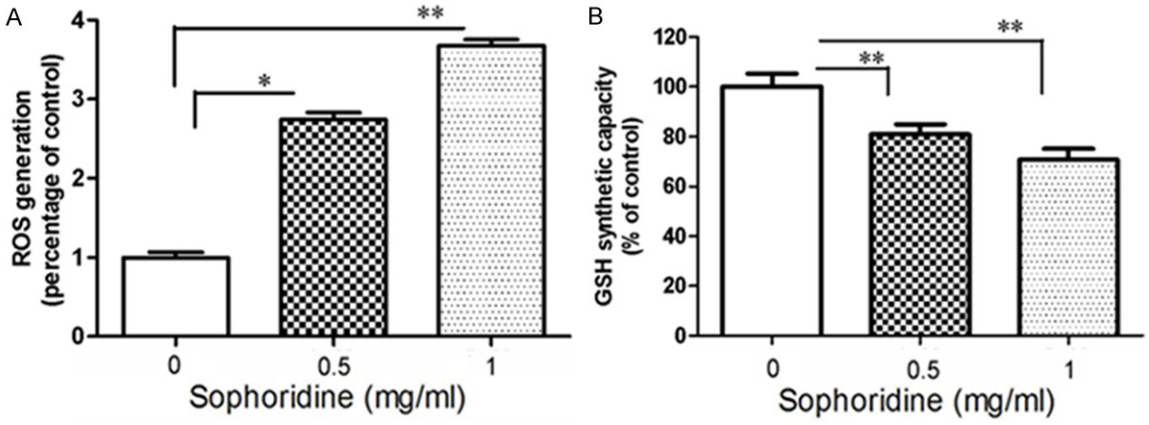

Figure 3. Sophoridine affected ROS production and GSH content in U87MG cells. A: ROS generation in U87MG cells treated with 0.5 mg/ml and 1 mg/ml Sophoridine was analyzed. B: Cellular GSH content. The GSH content assessed in cells that had been treated with 0.5 mg/ml and 1 mg/ml Sophoridine for 24 h was also analyzed as described. Bars mean ± SD. n = 3. The statistical significance was considered as *P < 0.05 and **P < 0.01 where compared with control.

Detection of the changes in tumor-related gene protein expression with Western blotting

U87MG were seeded in 6-well plates and cultured overnight to allow cell adhesion. Then, 0.5 mg/ml and 1 mg/ml of Sophoridine was added using PBS as the control to continue the culture for 24 h. Cells were harvest and lyse cells to extract the proteins. The protein content in cell lysate was determined with BCA assay; equal amount of protein was applied and separated with 12% SDS-PAGE; the proteins were then transferred onto PVDF membrane and incubated with the corresponding monoclonal antibody (p27, CDK2, Survivin, Livin, Bcl-2, E2F1, caspase-3/8, p53, Smac, c-JNK and p38-MAPK) at room temperature for 4 h to detect the target protein contents. After washing off the primary antibody, the membrane was incubated with HRP-conjugated secondary antibody at room temperature for 2 h. After several washes, ECL kit was used to reveal the immunoreactive bands. Beta-actin was used as an internal reference.

Detection of activity of ubiquitin-proteasome, FoxM1, NF-κb and AP-1 in tumor cells

The tumor cells of logarithmic growth phase were seeded in 96-well plates to continue the culture for 24 h. Ub-G76V-YFP plasmid (Add gene) was used to detect intracellular ubiquitin-proteasome activity, and the luciferase plasmid of FoxM1, NF-κb and AP-1 was used to detect their transcriptional activity. After 5 ng plasmid was added to each well, the transfection reagent (Life Science) was used to assist in the transfection for 24 h. The un-transfected plasmids were washed off and the culture medium was changed with fresh one. Then, PBS, 0.5 mg/ml and 1 mg/ml of Sophoridine was added to the respective test wells to continue the culture for 24 h. Finally, the fluorescence intensity of YFP protein in each well was detected with a fluorescence microplate reader.

Statistical analysis

The experimental data were presented as mean ± standard deviation and analyzed with SPSS 13.0 software. One-way ANOVA was employed for the comparison; When $P < 0.05$, the difference was considered to have statistical significance.

Results

Sophoridine significantly inhibited the growth of U87MG cells

MTS assay was used to evaluate the effect of Sophoridine on the in vitro growth of U87MG after treatment for 24 h. The test results showed that Sophoridine exerted significant inhibitory effect. The mean IC_{50} was 5.3 mg/ml for 24 h. See **Figure 1** for the representative results.

Sophoridine significantly enhanced apoptosis and arrested U87MG cells in G2/M phase

To evaluate the effect of Sophoridine on apoptosis, we selected 1 mg/ml and 2 mg/ml concentrations. The test results showed that 1 mg/ml Sophoridine mainly caused early-phase

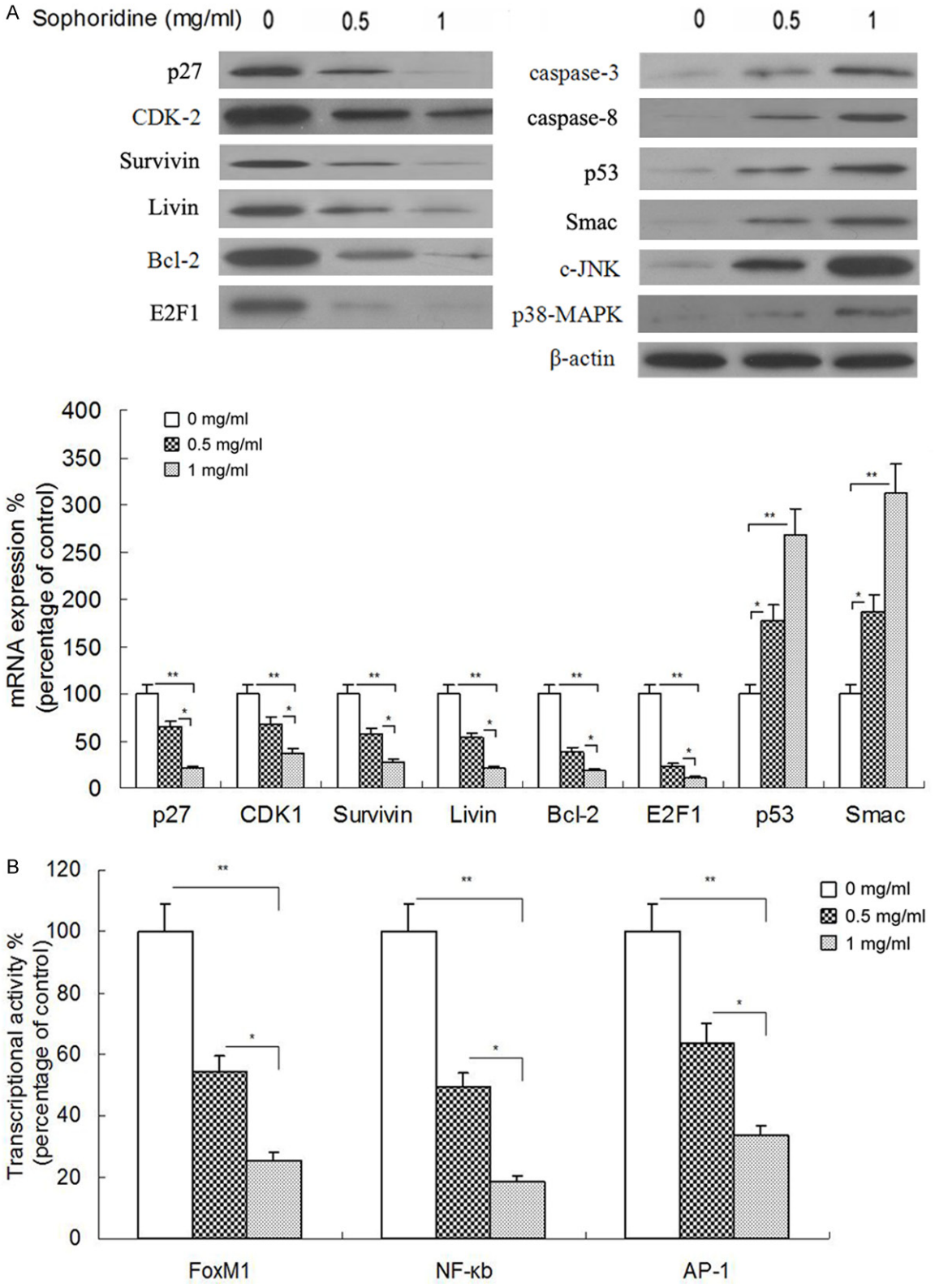

Figure 4. Sophoridine regulated tumor-related gene expression in U87MG cells. A: Immunoblotting assay and real-time PCR assay were measured the expression of tumor-related gene in protein and mRNA level. β-actin was as an internal control. Bars mean ± SD. n = 5. The statistical significance was considered as *P < 0.05 and **P < 0.01 where compared with control. B: Report gene assay for the transcriptional activity of FoxM1, NF-κb and AP-1. Bars mean ± SD. n = 5. The statistical significance was considered as *P < 0.05 and **P < 0.01 where compared with control.

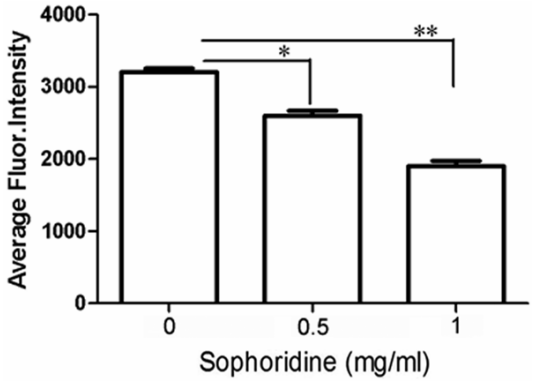

Figure 5. Sophoridine inhibited ubiquitin-proteasome activity in U87MG cells. Given cell lines were treated with 0.5 mg/mL and 1 mg/mL Sophoridine for the indicated time and then subjected to proteasome activity assay. Bars mean ± SD. n = 5. The statistical significance was considered as *P < 0.05 and **P < 0.01 where compared with control.

tumor cell apoptosis after treatment for 24 h while 2 mg/ml Sophoridine induced more late-phase apoptosis after treatment for 24 h. See **Figure 2B** and **2D** for the representative results. To study the effect of Sophoridine on cell cycle, we selected 0.5 mg/ml and 1 mg/ml of Sophoridine for the experiments (These concentrations of Sophoridine could help avoid excessive apoptosis). The test results showed that the cell cycle distribution changed significantly under the action of both concentrations of Sophoridine ($P < 0.05$); specifically, the cell cycle was arrested at G2/M phase. See **Figure 2A** and **2C** for the representative results.

Sophoridine induced ROS production and simultaneously caused reduction of GSH content in U87MG cells

Since one of the main mechanisms of Sophoridine's anti-tumor action is to induce the cells to produce ROS [3, 11], this study evaluated whether Sophoridine was able to increase the intracellular ROS level. The results showed that Sophoridine indeed increased ROS level in U87MG cells (**Figure 3A**). Further studies showed that the intracellular concentration of GSH for detoxifying ROS declined (**Figure 3B**), which further amplified the effect of ROS.

Sophoridine regulated tumor-related gene expression in U87MG cells

To further study the mechanism of Sophoridine-induced apoptosis and cell cycle arrest, we examined the changes in related genes. The results showed that Sophoridine triggered significant down-regulated the expression of p27, CDK2, Survivin, Livin, Bcl-2 and E2F1, meanwhile, up-regulated the expression of caspase-3/8, p53, Smac, c-JNK and p38-MAPK in protein and mRNA level (**Figure 4A**). Our study also found that Sophoridine down-regulated the transcriptional activity of FoxM1, NF-κb and AP-1 which was the key transcriptional factors in tumor related signaling pathways (**Figure 4B**).

Sophoridine inhibited ubiquitin-proteasome activity in U87MG cells

Because Ub-G76V-YFP, the expression product of Ub-G76V-YFP plasmid (Poulsen et al., 2012), is degraded continuously by intracellular ubiquitin-proteasome under normal circumstances, no or only very weak fluorescence of YFP protein can be detected. However, if ubiquitin-proteasome activity is inhibited, leading to the accumulation of Ub-G76V-YFP in the cells, intracellular YFP fluorescence will be enhanced (**Figure 5**). The experimental results showed that the fluorescence intensity was significantly higher in Sophoridine-treated group than in the control group, suggesting that Sophoridine significantly inhibited ubiquitin-proteasome in tumor cells.

Discussion

In this study, we first used MTS assay to examine whether Sophoridine was able to inhibit U87MG proliferation and measured an IC_{50} of 5.3 mg/ml, which was similar to the inhibition intensity of Sophoridine on other tumors as reported in the literature [5, 6]. Accordingly, we examined the apoptosis and cell cycle distribution of tumor cells in the subsequent experiments in order to unravel the patterns of Sophoridine-mediated inhibition of tumor growth. The results showed that Sophoridine significantly enhanced apoptosis at higher concentrations (1 and 2 mg/ml) and arrested tumor cell cycle arrest at G2/M phase at lower concentrations (0.5 mg/ml and 1 mg/ml). Western blotting was used to further study the molecular mechanism of Sophoridine-mediated tumor suppression. It was found that Livin, Survivin, Bcl-2 and E2F1, the anti-apoptotic protein in mitochondria-related apoptosis pathway [7-9] was down-regulated and the p53, Smac and caspase-3/8 was up-regulated by

Sophoridine, suggesting that the mitochondria pathway played an important role in Sophoridine-induced apoptosis. In addition, Western Blotting detection also revealed that the p27 and CDK-2 level in tumor cell decreased significantly after treatment with Sophoridine. Because p27 and CDK-2 are key regulatory protein after the tumor cells have entered mitosis, it suggests that Sophoridine arrested tumor cells in G2/M phase through down-regulation of p27 and CDK-2. The above results were consistent with the pattern of Sophoridine action in prostate cancer [10].

It is currently believed that one of the main mechanisms of Sophoridine's antitumor action is to induce the tumor cells to produce ROS that can subsequently kill the tumor cells and induce tumor apoptosis. In this study, we also found significant increase in ROS level and decrease in ROS-detoxifying GSH level in U87MG cells after treatment with Sophoridine, these factors can further amplify the actions of ROS in tumor cells. Furthermore, Sophoridine also could down-regulate the expression of Bcl-2 and surviving to prevent cancer cells from apoptosis [11]. In the studies on key proteins and important transcription factors in some of the key signaling pathways, we found that Sophoridine up-regulated the activity of c-JNK and p38-MAPK signaling pathways; the elevated activities in both pathways can inhibit the cell cycle and thereby inhibiting tumor cell growth [12]. In fact, JNK and p38 signaling pathway may play broader roles. As shown by some report, Sophoridine was able to up-regulate intracellular active free radicals by activating JNK and p38 in brain glioma cells and down-regulate the transcriptional activity of FoxM1, NF-κb and AP-1, leading to the kill of tumor cells.

Ubiquitin-proteasome plays a key role in the control of the content, activity and location of multiple intracellular proteins [13]. It can be observed in the majority of tumor cells that ubiquitin-proteasome is abnormally activated, leading to rapid degradation and inactivation of some of the tumor suppressors such as p53; therefore, targeted ubiquitin-proteasome is a potential antitumor therapy [14]. Bortezomib, a developed inhibitor of this proteasome has been successfully applied in the treatment of lymphoma and myeloma. It was found that natural products could inhibited ubiquitin-proteasome activity in tumor cells, including Sophoridine [15], which provided a new perspective for elucidating the mechanisms of Sophoridine's anti-tumor action. Likewise, this study also showed that Sophoridine significantly inhibited ubiquitin-proteasome activity in glioma cell U87MG. In our study showed that the inhibition of ubiquitin-proteasome was accompanied by ROS elevation. Therefore, inhibition of ubiquitin-proteasome activity may be the deeper level of molecular mechanism responsible for Sophoridine-induced ROS elevation in tumor cells.

Disclosure of conflict of interest

None.

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