A natural food sweetener with anti-pancreatic cancer properties

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Mogroside V is a triterpenoid isolated from the traditional Chinese medical plant Siraitia grosvenorii. Mogroside V has a high degree of sweetness and a low calorific content. Herein, we found that mogroside V possesses tumor growth inhibitory activity in vitro and in vivo models of pancreatic cancer by promoting apoptosis and cell cycle arrest of pancreatic cancer cells (PANC-1 cells), which may in part be mediated through regulating the STAT3 signaling pathway. These results were confirmed in vivo in a mouse xenograft model of pancreatic cancer. In xenograft tumors, Ki-67 and PCNA, the most commonly used markers of tumor cell proliferation, were downregulated after intravenous administration of mogroside V. Terminal deoxynucleotidyl transferase dUTP nick end labeling assays showed that mogroside V treatment promoted apoptosis of pancreatic cancer cells in the xenograft tumors. Furthermore, we found that mogroside V treatment significantly reduced the expression of CD31, labeled blood vessels and of the pro-angiogenic factor vascular endothelial growth factor in the xenografts, indicating that mogroside V might limit the growth of pancreatic tumors by inhibiting angiogenesis and reducing vascular density. These results therefore demonstrate that the natural, sweet-tasting compound mogroside V can inhibit proliferation and survival of pancreatic cancer cells via targeting multiple biological targets.

INTRODUCTION

Cancer poses an important threat to human health, and seriously affects the quality of life. For example, patients with cancer experience fatigue, weakness and high levels of pain. In addition, chemotherapy drugs used in the treatment of cancers have numerous side effects that also seriously affect the quality of life. Therefore, it is particularly important to increase the efficiency of cancer drugs and reduce the side effects of chemotherapy to ensure a sustainable and effective chemotherapy course.

Chemotherapy is one of the most important antitumor therapies. Chemotherapeutic drugs are greatly improving the quality of life and prolonging survival, and thus, research for high efficiency chemotherapy drugs with low toxicity is a major focus of oncology research. Although the screening of pharmacologically active compounds in plants is an important approach for developing new drugs, plants that may contain pharmacologically active compounds are widely distributed and numerous, and the compounds are complex in abundance, random screening of plant-derived drugs is laborious. Traditional Chinese medicine uses plants that are proven to be clinically effective for thousands of years, thereby providing a large resource for drug development.¹² Therefore, screening single compounds that have particularly high efficiency in Chinese herbal medicines may be an important approach for developing new therapies.

Among the plants commonly used in traditional Chinese medicine is Siraitia grosvenorii, an endemic plant in China that is mainly grown in Guangxi province, which accounts for more than 90% of the global S. grosvenorii production. In 1997, the Chinese Ministry of Health approved S. grosvenorii saponins as a sweetener in various types of foods. S. grosvenorii saponins are the main sweetening compounds in S. grosvenorii and are several hundredfold sweeter than sucrose.

S. grosvenorii is among the first group of ‘Medicinal and Edible’ drugs included in the Pharmacopoeia of the People’s Republic of China,³ which lists the main effects of S. grosvenorii as ‘heat-releasing and lung-moistening, beneficial to the pharynx and voice, facilitating to bowel movement and acting as a laxative.’ Currently, studies on S. grosvenorii are focused on its antioxidant,⁴,⁵ anti-inflammatory,⁶ and blood lipid- and sugar-reducing potential.⁷ However, thus far, the numerous studies on S. grosvenorii have not been sufficiently comprehensive. Notably, they have failed to provide a clear mechanism of action and to characterize the full pharmacological effects of this medicinal plant. In particular, the use of S. grosvenorii in the development of anti-pancreatic cancer drugs has not been explored.

Pancreatic cancer is a malignant disease that affects glucose metabolism.⁸,⁹ Thus, pancreatic cancer patients should avoid excessive sugar intake, as chronic excessive consumption of sugar increases the risk of pancreatic cancer. However, most people consume sweet foods and drinks in everyday life. Thus, a means to provide anticaner treatment along with a clinically approved sweetener would be particularly beneficial for pancreatic cancer patients. Herein, it was observed that mogroside V extracted from S. grosvenorii inhibited pancreatic cancer cell proliferation and survival through the STAT3 pathway both in vivo and in vitro.
These results indicate that mogroside V may be a promising anticancer drug for daily use with relatively few side effects.

RESULTS

Mogroside V inhibited the proliferation and survival of PANC-1 cells

We examined the effects of mogroside V (Figure 1a) on the proliferation of PANC-1 cells and other tumor types in liquid cell culture using the MTT assay. As shown in Figure 1b, mogroside V inhibited the proliferation of pancreatic cancer cells and other tumor cell types in a dose- and time-dependent manner, and showed very little cytotoxicity against the non-tumorigenic epithelial cell line L02. To further determine whether the anti-proliferative effects of mogroside V were related to the induction of apoptosis and/or necrosis, mogroside V-treated cells were analyzed with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. After 24 h of treatment with mogroside V, the percentage of TUNEL-positive cells was increased in a concentration-dependent manner, ranging from 2.91% in the non-treated control cells to 92.25% in the cells treated with 250 μmol/l of mogroside V (Figure 1c).

Mogroside V induced apoptosis of PANC-1 cells and caused cell cycle arrest at the G0/G1 phase

To further investigate the inhibition of cell proliferation and viability by mogroside V, apoptosis and the cell cycle distribution of PANC-1 cells treated with mogroside V concentrations ranging from 0 to 250 μmol/l were examined using flow cytometry. Annexin V flow cytometry assays revealed that mogroside V caused apoptosis in PANC-1 cells. As illustrated in Figures 2a and b, mogroside V induced apoptosis in PANC-1 cells in a concentration- and time-dependent manner. We further found that z-VAD-fmk, a general caspase inhibitor, inhibited apoptosis of PANC-1 cells induced by treatment with mogroside V for 24 h (Figure 2c). Furthermore, a mogroside V concentration-dependent increase in the G0/G1 ratio was observed, reaching a 1.7-fold increase after exposure to the highest dose (250 μmol/l) compared with that in the untreated cells (Figure 2d).

Mogroside V altered the level of STAT3 signaling activity

The STAT3 pathway has an important role in cell growth, proliferation and survival, and is implicated in several types of human cancer. We therefore assessed the effect of mogroside V treatment on this signaling pathway in PANC-1 cells by western blot assays. We found that the level of phosphorylated STAT3 (p-STAT3) in PANC-1 cells was reduced after treatment with mogroside V for 24 h (Figure 3). We further examined whether mogroside V modulated the expression of proteins involved in cell cycle regulation and apoptotic pathways that are downstream of the STAT3 pathway. The expression of the pro-apoptotic proteins CDKN1A (p21), CDKN1B (p27), which are associated with cell cycle arrest in the G0/G1 phase, was increased by mogroside V in a dose-dependent manner after 24 h. In contrast, the expression of the pro-proliferative cell cycle regulators CCND1 (cyclin D1), CCNE1 (cyclin E1), and CDK2 was decreased after mogroside V treatment. Furthermore, the expression of the anti-apoptotic proteins BCL2 and BCL2 was decreased, whereas that of the proapoptotic CASP3 and BAX proteins was increased after the 24-h mogroside V treatment. In addition, mogroside V suppressed phosphorylation of the kinases upstream of STAT3, including JAK2 and TYK2 (Figures 3c and d).

Evaluation of the antitumor activity of mogroside V in vivo

To determine the ability of mogroside V to inhibit tumor growth in vivo, we evaluated the antitumor activity of mogroside V against PANC-1 tumors in a nude mouse xenograft model by intravenously injecting experimental mice with different doses of mogroside V 3 days/week for 5 weeks. Tumor growth was significantly inhibited in the mice treated with mogroside V at all doses evaluated compared with that in the control mice (Figure 4a). The mice were killed and the tumors were anatomized.

Figure 1. Mogroside V inhibits the proliferation and induces apoptosis in cultured cells. (a) Chemical structure of mogroside V extracted from S. grosvenorii. (b) Graph of MTT assay results indicating the rate of cell proliferation inhibition in PANC-1 and other tumor cell types treated with mogroside V. (c) Representative images of cells plated onto slides for TUNEL reaction apoptosis assays. The percentage of cells undergoing apoptosis increased with increasing mogroside V concentrations. The results were obtained in three independent experiments.
35 days after xenografting. The mean tumor volume in the control mice was 278.6 ± 0.03 mm$^3$ compared with 56.4 ± 0.11 mm$^3$ in the high-dose mogroside V-treated mice (79.7% decrease; $P < 0.001$). The overall tumor weight in the control group was 32.2 ± 1.4 g, whereas it was only 9.2 ± 1.8 g in the mice that had received the high-dose mogroside V treatment (71.4% decrease; Figure 4b).

Overall survival rates were estimated by the Kaplan–Meier method, confirming the association between mogroside V treatment and survival (see Figures 4c for the survival curve). Our data demonstrated that mogroside V could not only increase survival of the mice used for the in vivo pancreatic cancer model but also that of the mice used for the in vivo colon cancer HT29...
and laryngeal cancer HEP-2 xenograft models (Supplementary Figure S1). The results of these xenograft experiments were consistent with the in vitro data shown in Figure 1b, and indicated that mogroside V might be a potent tumor growth inhibitor.

Immunohistochemistry analysis (IHC) of tumor cell proliferation, apoptosis and angiogenesis

The potential mechanisms underlying tumor growth inhibition by mogroside V in vivo were assessed with IHC assays detecting cell death, cell proliferation and angiogenesis. TUNEL staining of tissue sections (Figure 5) revealed that more cells were TUNEL-positive in the low-dose (57.8% TUNEL-positive), moderate dose (73.1% TUNEL-positive) and high-dose (82.1% TUNEL-positive) mogroside V groups compared with cells in the control group (5.2% TUNEL-positive), indicating that mogroside V induced a high rate of apoptosis in PANC-1 tumor cells.

We next assessed cell proliferation in the tumors using IHC to detect PCNA and Ki-67. The number of cells expressing these markers was significantly decreased in all mogroside V treatment groups compared with that in the control groups (P < 0.001) (Figure 6). PCNA and Ki-67 mRNA expression in vivo was evaluated by real-time–PCR (RT–PCR) assays (Figure 6). Mogroside V clearly reduced PCNA and Ki-67 mRNA expression, which was in accordance with the IHC data shown in Figure 6 (P < 0.001). Taken together, these data indicated that mogroside V inhibited tumor cell survival and proliferation.

Finally, we determined whether mogroside V affected microvascular density (MVD) and angiogenesis in tumors. As shown in Figure 7a, expression of the pro-angiogenic factor vascular endothelial growth factor (VEGF) was lower in the mogroside V treatment groups (22.82% of the cells was VEGF+ in the highest dosage group) than that in the control group (64.71% of the cells was positive; P < 0.001); thus, there were 64.73% fewer...
VEGF-expressing cells after treatment with the highest dose of mogroside V. To examine the effect of mogroside V treatment on MVD in the tumors, blood vessels were labeled with CD31 (Figure 7b) and MVD was calculated according to Weidner’s method. The MVD value (Figure 7c) in the mogroside V-treated group (4.11) was significantly lower than that in the control group (37.23; 88.96% decrease, \( P < 0.001 \)). These results showed that mogroside V inhibited tumor angiogenesis in a dose-dependent manner.

**DISCUSSION**

In this study, a cellular and molecular approach was used to determine the mechanisms underlying cell division and PANC-1 tumor survival regulation by mogroside V. Our findings clearly demonstrated that mogroside V treatment inhibited tumor cell growth, induced apoptosis and caused tumor regression. Moreover, mogroside V inhibited the proliferation of not only pancreatic cancer cells but also that of U937 and A549 cancer cells. In contrast, mogroside V at equivalent concentrations had a minimal effect on normal human liver cells (L02) (Figure 1b). Taken together, these results suggest that mogroside V may be less toxic to normal cells than to cancer cells.

Heretofore, the biochemical targets of mogroside V had not been elucidated. Herein, using western blotting, we demonstrated that mogroside V regulated the protein expression of STAT3. Moreover, mogroside V inhibited the downstream targets of the STAT3 pathway that promote cell proliferation (CCND1, CCNE1 and CDK2), while also upregulating cell cycle inhibitors (CDKN1A and CDKN1B). These effects are in accordance with our finding of a dose-dependent G0-G1 cell cycle arrest in mogroside V-treated PANC-1 cells.\(^{17}\) We further found that mogroside V treatment inhibited anti-apoptotic signals downstream of STAT3 (for example, BCL2 and BCL2L1),\(^{18,19}\) which coincided with an increase in the expression of the proapoptotic proteins CASP3 and BAX.\(^{20,21}\) We further found that apoptosis of PANC-1 cells was partially blocked by the caspase inhibitor z-VAD-fmk (Figure 2c), suggesting that mogroside V-induced apoptosis may in part be mediated through the caspase pathway.

Our results obtained using the PANC-1 xenograft models indicated that mogroside V potently inhibited tumor growth \textit{in vivo}. Furthermore, similar to our \textit{in vitro} findings, mogroside
Figure 5. Immunohistochemical analysis of PANC-1 cell xenograft tumor apoptosis. Cells were stained using the TUNEL method and were scored for apoptosis by comparison with the vehicle control. The images on the left illustrate the level of TUNEL staining in the PANC-1 cells comprising the tumor in the control and mogroside V treatment groups. The graphs on the right indicate a mogroside V concentration-dependent increase in TUNEL staining as determined by counting tissue sections from eight independent tumors. For statistical analysis, mogroside V-treated mice were compared with vehicle control mice using Student’s t-tests; ***P < 0.001 versus the control values.

Figure 6. The effect of mogroside V on the expression of PCNA and Ki-67 in PANC-1 xenograft tumors. (a) Images of immunohistochemical staining and RT–PCR analysis indicating that mogroside V inhibits PCNA expression. (b) Images of immunohistochemical staining and RT–PCR analysis indicating that mogroside V inhibited PCNA expression. The data represent the means ± s.d. Statistical analysis was performed using the Student’s t-test; ***P < 0.001 versus the control values.

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V also reduced cell viability and proliferation in vivo and reduced p-STAT3 in PANC-1 tumors in a dose-dependent manner (Supplementary Figure S2). TUNEL assays revealed that mogroside V strongly induced apoptosis. Proliferative activity in xenograft tumors was assessed by IHC for Ki-67 and PCNA. Ki-67 is a cell proliferation marker that is expressed in all phases of the cell cycle, except the G0 phase. PCNA, a 36-kD DNA polymerase delta auxiliary protein involved in the proliferation of neoplastic and non-neoplastic cells, is specifically expressed in proliferating cell nuclei and is commonly used to measure tumor cell proliferation. The expression of both Ki-67 and PCNA was reduced in PANC-1 xenograft tumors in all mogroside V treatment groups compared with that in the control groups and corresponded with reductions in tumor weight. Thus, mogroside V may act pharmacologically by inhibiting the proliferation of PANC-1 cells, while also inducing apoptosis, thereby subsequently limiting pancreatic tumor growth.

Figure 7. Determination of angiogenesis by immunohistochemical analysis of VEGF and CD31 expression. VEGF and CD31 protein expression was significantly downregulated in PANC-1 tumors. (a) Immunohistochemical staining of VEGF in xenograft tumors of each of the indicated control and mogroside V treatment groups. (b) Representative images of CD31 immunohistochemical staining in xenograft tumors of each of the indicated control and mogroside V treatment groups. (c) Graph of the MVD measurements in sections of xenograft tumors as determined by CD31 expression. The data represent the means ± s.d. Statistical analysis was performed using the Student’s t-test. *P < 0.05, **P < 0.01 and ***P < 0.001 versus the control values.
role in angiogenesis in both tumors and healthy tissues.26,27 These data thus suggest that mogroside V may prevent pancreatic tumor growth by inhibiting angiogenesis through a VEGF-dependent mechanism. Consequently, this would lead to decreased MVD and, hence, diminished tumor growth. STAT3 is an essential mediator mechanism. Consequently, this would lead to decreased MVD and, the PANC-1 cells used as tumor xenografts. The effects of a stable overexpression of STAT3 or deletion of STAT3 in prevent STAT3 activation.

Our study was limited by the fact that we did not explore the effects of a stable overexpression of STAT3 or deletion of STAT3 in the PANC-1 cells used as tumor xenografts. The efficacy of mogroside V treatment under these conditions is not known and will be the focus of our future studies.

In conclusion, we have found through in vitro and in vivo experiments that mogroside V inhibited pancreatic cancer cell proliferation and promoted tumor apoptosis. Moreover, mogroside V interfered with angiogenesis in pancreatic tumors. Therefore, our results suggest that mogroside V may be a potential antitumor compound that acts on the STAT3 pathway. Moreover, we demonstrated that mogroside V could not only prevent tumor progression in vivo but also increase survival of the mice, indicating that this natural compound might possess potential therapeutic activity. Given that mogroside V is a natural sweetener that has been approved by the United States Food and Drug Administration, it may be used for daily consumption as an additive in foods and drinks to prevent or treat pancreatic cancer.

MATERIALS AND METHODS

Drugs and reagents
Mogroside V was purchased from Sigma-Aldrich (St Louis, MO, USA). All other reagents were also purchased from Sigma-Aldrich unless specified otherwise.

Cell lines and cell culture
The human pancreatic adenocarcinoma cell line PANC-1 was obtained from Shanghai Cell Biology Institute (Shanghai, China). PANC-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FCS; Gibco, Grand Island, NY, USA), 100 μg/ml streptomycin and 100 U/ml penicillin under a humidified 5% CO2 atmosphere at 37°C. Human pancreatic adenocarcinoma cell lines MiaPaCa-2 and CPFPAC-1, As49 lung adenocarcinoma, human U937 and L02 normal human liver cells were obtained from Shanghai Cell Biology Institute. MiaPaCa-2 and CPFPAC-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 μg/ml streptomycin and 100 U/ml penicillin. The cells were maintained at 37°C with 5% CO2.

In vitro cellular proliferation (MTT) and TUNEL assays
Cells were seeded into 96-well cell culture plates at an initial concentration of 1 x 103 cells/ml and were incubated with the indicated concentrations of mogroside V for 24 or 72 h of incubation, MTT solution (5 mg/ml) was added to the wells and the cells were incubated for another 4 h. The growth medium was removed and formazan crystals formed by oxidation of the MTT dye were analyzed by measuring the absorbance at 490 nm using an ELISA reader.

Cells were labeled for apoptotic DNA strand breaks by a TUNEL reaction using the In Situ Cell Death Detection Kit (POD; Roche Applied Science, Indianapolis, IN, USA) to determine the percentage of cells undergoing mogroside V-induced apoptosis, and were incubated with antioxidigenic peroxidase for 30 min using DAB substrate. The apoptotic cells were visualized using light microscopy (Olympus, Tokyo, Japan). For quantifying apoptotic cells, the percentage of TUNEL-positive cells in a cancer cell population of 300 cells was determined in five random fields per glass slide.

Cell cycle analysis and measurement of apoptosis
Cell cycle status was determined using flow cytometry. PANC-1 cells were analyzed for alterations in their cell cycle after treatment with mogroside V for 24 h. The cells used for the flow cytometry experiments were both floating and adherent cells. After the cells were fixed with 70% methanol, they were treated with RNase A and were exposed to propidium iodide for flow cytometry analysis. Detection of apoptosis was performed using Annexin V-FITC staining with an Annexin V assay kit (Annexin V-FITC Apoptosis Detection Kit; BD Pharmingen, San Diego, CA, USA). Briefly, PANC-1 cells (1 x 105 cells) were cultured with mogroside V for 24 to 96 h, followed by labeling with Annexin V-FITC and propidium iodide according to the manufacturer's instructions. The percentage of cells undergoing apoptosis in the control and mogroside V-treated cells was analyzed using flow cytometry. In the apoptosis inhibition assays, cells were incubated with or without mogroside V for 48 h in the presence or absence of the apoptosis inhibitor zVAD (20 μmol/l). The results were evaluated with the CellQuest software (BD Biosciences, San Jose, CA, USA).

Western blot assay
Cells were treated with mogroside V for 24 h and were lysed at 4°C in lysis buffer (0.05 M Tris-HCl, pH 7.4, 1% Triton X-100, 0.1 M NaCl, 1% sodium deoxycholate and 0.1% SDS) containing 1 mm phenylmethylsulfonyl fluoride and a protease inhibitor. The insoluble protein lysate was removed by centrifugation. About 15 μg of protein from the cell lysates were separated by 10% SDS-PAGE and were subsequently transferred onto a polyvinylidene fluoride membrane. The following primary antibodies were used for western blot analysis: STAT3 (1:1000 dilution), phospho-(p)-STAT3, CDKN1A, CDKN1B (p27), BCL2 and B-actin (all from Sigma-Aldrich). After incubation with the appropriate secondary antibodies (all from Sigma-Aldrich), the membranes were developed with ECL plus (ECL, Amersham Biosciences, GE Healthcare, Pittsburgh, PA, USA). Densitometric measurements of the protein bands were performed using the GS-800 imaging densitometer (Bio-Rad, Hercules, CA, USA).

PANC-1 xenograft model and tumor treatment
The in vivo characteristics of the pancreatic tumors were investigated in a BALB/c nude mice xenograft model. Approximately 8-week-old male nude BALB/c mice from Vital River (Charles River Ltd. Co., Beijing, China) were maintained in a pathogen-free environment. To establish a xenograft tumor model, PANC-1 cells (1 x 105) were injected subcutaneously in the flank of the mice. The mice were randomly divided into the following five groups: (i) untreated control group, (ii) NC group (normal saline), (iii) 2 mg/kg b.w. mogroside V group, (iv) 10 mg/kg b.w. mogroside V group and (v) 30 mg/kg b.w. mogroside V. Mogroside V was dissolved in normal saline and was administered to the mice in a volume of 0.1 ml. The tumors were measured with a vernier caliper every 3 days. The tumor volumes were determined by measuring the length (l) and width (w) of the tumors, and by calculating the tumor volume (V = l x w2). After 5 weeks, the mice were killed and the tumors were dissected and weighed. The inhibition rate of tumor growth was calculated using the following formula: tumor growth inhibition rate (%) = (1 - Mt/M0) x 100, where Mt and M0 are the mean tumor masses of the treatment and control groups, respectively.

Animal use complied with Beijing University of Agriculture Animal Welfare Guidelines.

Immunohistochemistry
Following dissection, the tumor tissues were fixed immediately in 10% phosphate-buffered formalin and were embedded in paraffin. Proliferation of pancreatic cancer cells was estimated by IHC staining with either a mouse monoclonal PCNA or Ki-67 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and the number of PCNA- and Ki-67-positive cells in a population of 200 cells sampled at x400 magnification was counted. The proliferating cell nuclear antigen labeling index and Ki-67 labeling index were subsequently calculated. After treatment with mogroside V, vascularity was evaluated in CD31- and VEGF-immunostained tissue sections. The MVD was calculated according to Weidner's method. TUNEL immunohistochemistry was performed using an In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

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Quantitative real-time PCR (RT–PCR) analysis

The expression levels of the mRNA transcripts of interest were determined by RT–PCR assays. Total RNA was isolated from fresh tumor tissues with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Oligo(dT)-primed RNA (2 μg) was reverse-transcribed with SuperScript II reverse transcriptase (Promega, Madison, WI, USA). GAPDH was used as an internal control. The following primer sequences were used for amplification of PCNA: Ki-67 and GAPDH. PCNA forward 5′-CGCTGTTGGATATACTGCCTA-3′ and reverse 5′-CAGCGGTAGTTGCTGACACG-3′ (Tm = 60 °C, 109 bp); Ki-67 forward 5′-GCTGCTGACCTACAGA-3′ and reverse 5′-GCTGTCAAGCGTGTCG-3′ (Tm = 60 °C, 127 bp); GAPDH forward 5′-CTGGGCTACATGCAGCAC-3′ and reverse 5′-AAGTGGTCGGTGGCAAGACT-3′ (Tm = 60 °C, 101 bp). The resultant PCR samples were analyzed by gel electrophoresis (1.5% agarose). The DNA bands were examined using a Gel Documentation System (Model Gel Doc 2000; Bio-Rad, Hercules, CA, USA).

Statistics

The data were presented as the means ± s.d. of three independent experiments for the in vitro study and n = 6 for the in vivo study. Statistical significance was determined using Student’s t-test. Survival was estimated using the Kaplan–Meier method and the values obtained were compared using the log-rank test. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) with P < 0.05 considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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