Sinomenine inhibits the growth of glioma cells through STAT3 signal pathway

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\begin{abstract}
The present study was designed to examine effects of Sinomenine (SM) on glioma cells growth \textit{in vivo} and \textit{in vitro}. Cells growth and apoptosis were detected by MTT assay, TUNEL assay and flow cytometric analysis. In the study, SM treatment led to growth inhibition on a series of glioma cell lines, including U87, U373, U251, Hs683 and T98G. SM prevented U87 growth in the nude mice as well. Inhibitory effects of SM on U87 cells proliferation \textit{in vitro} and \textit{in vivo} were more effective than that of temozolomide (TMZ), and SM has synergistic effects with TMZ in the glioma therapy. SM induced apoptotic death in U87 cells via activation of caspase-3, caspase-8 and caspase-9, and down-regulation of HIAP, Bcl-2 and survivin. Moreover, we observed SM decreased the expression of phosphorylated STAT3 (p-STAT3) both \textit{in vivo} and \textit{in vitro}. Interestingly, using a specific activator of STAT3, we demonstrated overexpression of p-STAT3 impaired, SM mediated growth inhibition and apoptosis induction in the U87 cells. In summary, our results indicate SM induced growth suppression of human glioma cells through inhibiting phosphorylation of STAT3.

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\section*{Introduction}

Glioblastoma (GBM) is one of the most common types of malignant primary brain tumours and has the worst prognosis (Stupp et al., 2010). Currently, the gold standard of therapy for newly diagnosed GBM is a surgical resection followed by radiotherapy plus adjuvant temozolomide (TMZ) (Stupp et al., 2005). However, it is difficult to remove all the tumour cells completely due to their resistance to radiotherapy and TMZ. This may lead to a relapse of the residual nidus, resulting in high recurrence and low cure rates (Van Meir et al., 2010). In addition, the patients usually are intolerable to TMZ therapy for the serious side effects (Van Meir et al., 2010). Therefore, the development of a more effective and safe therapeutic agent is urgently needed.

Sinomenine (SM, Fig. 1A) is a naturally occurring alkaloid enriched in stems and roots of \textit{Sinomenium actum} Rehd. et Wils. Modern pharmacological studies have shown that SM has cardioprotective, anti-inflammatory activities and cancer chemopreventive property (Nishida and Satoh, 2007; Wang and Li, 2011). Recently, a large number of studies indicated the beneficial roles of SM against several types of cancers, including breast cancer cells (Li et al., 2014), esophageal carcinoma cells (Wang et al., 2013), hepatocellular carcinoma cells (Lu et al., 2013), gastric adenocarcinoma cells (Lv et al., 2011), lung cancer cells (Zhou et al., 2012) and colon carcinoma cells (Yang et al., 2016). However, the molecular mechanisms of anti-tumour effects by SM are still not clarified.

Signal transducer and activator of transcription3 (STAT3), mediated by several cytokines (such as interleukin-6: IL-6), plays an important role in the regulation of cell proliferation, cell cycle progression and apoptosis (Nie et al., 2015). Once stimulated by...
cytokines, activated STAT3 (phosphorylated-STAT3: p-STAT3) induces the expression of genes that participate in oncogenesis, such as anti-apoptosis proteins (HIAP-1, Bcl-2 and survivin) (Banerjee and Resat, 2016; Nie et al., 2015). Interestingly, high levels of STAT3 correlated with reduced survival time and poor prognosis in glioma patients (Rodrigues et al., 2016; Xu et al., 2016). Therefore, it is suggested that STAT3 is a promising target for glioma therapy.

To the best of our knowledge, the effects of SM on glioma are still unclear. In this study, the effect of SM on the growth of human glioma cells was investigated, and an attempt was made to study its mechanism of action by exploring the relationship between the effects of SM and STAT3.

Materials and methods

Cell lines and culture conditions

Human glioma cell lines (U87, U373, U251, Hs683 and T98G) and the rats astrocytes cell line (RA) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were routinely cultured in Dulbecco’s modified eagle’s medium (Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc.), 100 U/ml penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified incubator with 5% CO2 at 37°C.

MTT assay

The effects of SM on the growth of human glioma cell lines and RA were determined using MTT assay. In short, the cells were cultured in 96-well plates with a density of 5 x 103/well, and then treated with SM (purity beyond 98%, Xi’an Sobeo Pharmtech co., Ltd. Xi’an, China) or TMZ (purity beyond 98%, Sigma-Aldrich, St. Louis, MO, USA) for the designated time (24–72 h). Following incubation, 10 μl MTT solution (5 g/l, Sigma-Aldrich, St Louis, MO, USA) was added to the medium in each well, and the microplate was incubated at 37°C for 4 h. The absorbance was read in a microplate reader at 570 nm (Bio-Rad, Hercules, CA, USA). Cytotoxicity was expressed as the percentage of cells surviving in relation to untreated cells, and was calculated using following formulas: Viability = (A570drug/A570DMSO) x 100%; Inhibition rate (%) = 100% – viability (%). The drug concentration required to inhibit growth by 50% (IC50) was calculated from dose-response curves fitted by GraphPad Prism® software (GraphPad Prism® 5.01, GraphPad Software, Inc., CA, USA).

Apoptosis assay

Apoptosis was analyzed using FITC/PI kit (Nanjing KeyGen Biotech Co., Ltd, Nanjing, China) according to the manufacturer’s recommendations. Briefly, the cells were seeded in the plates with a density of 5 x 103/well, and incubated with SM (50 μM, 100 μM) for 48 h. The cells were then washed three times in PBS buffer and re-suspended in the binding buffer. Following that, samples were incubated with 5 μl Annexin-V FITC and 5 μl PI for 15 min at room temperature, and finally analyzed by flow cytometry (FACScan, Becton Dickinson, USA). Cells in the earlier stages of apoptosis were stained positive for annexin V-FITC, whereas those in the later stages were stained positive for both annexin V-FITC and PI.

TUNEL assay

U87 cells were treated with SM (100 μM) in the absence or presence of IL-6 (20 ng/ml, PeproTech, USA) for 48 h. A TUNEL assay was conducted according to the manufacturer’s instruction (Promega Corporation, Madison, WI, USA). A DAPI filter was used to detect DAPI staining (blue colour) and an FITC filter was used to detect TUNEL staining (green colour). TUNEL-positive and DAPI-positive staining patterns were obtained by a confocal laser
scanning microscope (TCS SP8, Leica Microsystems, Germany). TUNEL-positive cells in different area of each slide were counted by an observer who was blinded to the treatment conditions.

**Caspases activation assay**

U87 cells were seeded (5 × 10³ cells/well) in the 96-well plates, and treated with SM (50 μM, 100 μM) for 48 h. The cells were then collected and added to 100 ml lysis buffer on ice for 15 min. After centrifugation (16,000–20,000g, 4°C, 15 min), the supernatant was collected and stored at -80°C for caspases activity measurement. Activities of caspase-3, caspase-8 and caspase-9 were assessed according to instructions of colorimetric assay kits provided by manufacturer (Beyotime Institute of Biotechnology, Haimen, China).

**Western blot analysis**

U87 cells were treated with SM (50 μM or 100 μM) for 48 h. After treatment, the cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China). Proteins were quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology). Protein samples were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with antibodies of HIAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-2 (Santa Cruz), survivin (Santa Cruz), STAT3 (Proteintech, Chicago IL, USA) and p-STAT3 (Proteintech) overnight at 4°C, and then labelled with the horseradish peroxidase conjugated secondary antibody for 2 h at room temperature. The immunoblots were visualized using a chemiluminescence detection kit (Pierce Chemical, Rockford, IL, USA). Moreover, the expression of HIAP, Bcl-2 and surviving in tumour tissues was detected by the same method. GAPDH was used as an internal control for relative quantification.

**Immunohistochemistry**

Tumour tissue from the nude mice were deparafinized and rehydrated, followed by treatment with 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity. After boiling in 10% citrate buffer to unmask antigens, the sections were incubated with primary antibody of p-STAT3 (Proteintech). After washing with TBS-Tween, sections were incubated with secondary antibody for 45 min at room temperature. Images were collected by Olympus light microscopy (Olympus, Japan), and at least five visual fields in each section were randomly selected for calculating the positive numbers. Over 500 cells were counted to calculate the mean percentage, which meant the percentage of stained cells.

**Xenograft mouse model**

Nude mice (male, 5-week-old BALB/c) were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The present study was approved by the Ethical Committee on animal care and use of LongSai hospital (Ningbo, China), and was complied with the ARRIVE guidelines and carried out in accordance with the U.K. Animals (Scientific Procedures) Act. Xenograft tumours were generated by injecting U87 cells (1 × 10⁷ for each), subcutaneously on flanks of all nude mice. After tumour formation (average temperature. The immunoblots were visualized using a chemiluminescence detection kit (Pierce Chemical, Rockford, IL, USA). Moreover, the expression of HIAP, Bcl-2 and surviving in tumour tissues was detected by the same method. GAPDH was used as an internal control for relative quantification.

**Fig. 2.** Effects of SM on apoptosis of U87 glioma cells. A: representative pictures of flow cytometry; B: the percentages of total apoptosis, early apoptosis and late apoptosis in U87 cells treated with SM. The data were obtained from three independent experiments and were shown as mean ± SD; **P < 0.01, compared to the control group without SM treatment.
volume of 200 mm$^3$), all mice were randomly divided into four groups (Xu et al., 2008; Yang et al., 2016; Yu et al., 2015): control group, SM group (100 mg/kg), TMZ group (20 mg/kg) and the combined group (SM 50 mg/kg + TMZ 10 mg/kg). Mice were given daily by intraperitoneal injections with normal saline, SM and TMZ for three weeks. During treatment, the weight of mice and the tumours sizes were measured every 3 days. Tumour size ($V$) was determined using the following equation: $V = (X \times Y)^2 / 2$, in which $X$ means the maximum diameter, and $Y$ represents the minimum diameter. At the end of the experiment, mice were sacrificed by cervical dislocation and tumours were removed. Tumours were weighted and then used for immunohistochemical analysis. Moreover, xenograft tumour in mice was established again to study effects of SM on the survival time and survival quality.

Fig. 3. Effects of SM on caspases activities and anti-apoptotic proteins expression in U87 cells. A1: caspase-3; A2: caspase-8; A3: caspase-9; B1: HIAP; B2: Bcl-2; B3: survivin; B4: representative protein bands of HIAP, Bcl-2 and survivin. The results were obtained from three independent experiments and were shown as mean ± SD; **$P < 0.01$, compared to the control group or U87 group.

Fig. 4. SM and TMZ synergistically inhibit U87 cells growth in vivo. A: tumour volume; B: tumour weight; C: survival time of tumour mice was recorded and represented in a Kaplan–Meier plot; D: body weight; the data were shown as mean ± SD, $n = 5$–10; **$P < 0.01$, compared to the control group; #$P < 0.05$, ##$P < 0.01$, compared to the TMZ (or SM) group alone.
All results are expressed as mean ± SD. Data analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences were analyzed using one-way ANOVA and Student’s t-test. A value of \( P < 0.05 \) was considered statistically significant.

**Results**

**SM inhibits the growth of glioma cell lines in vitro**

To determine effects of SM on the growth of glioma cells, several human glioma cell lines were used. As shown in Fig 1B, C, SM treatment resulted in dose- and time-dependent growth inhibition in U87, and IC50 of SM ranged from 178 \( \mu \text{M} \) to 380 \( \mu \text{M} \). Moreover, treatment of U251, U373, Hs683 and T98G with SM for 48 h obviously decreased cells viability, and IC50 were 342.7 \( \mu \text{M} \), 430.2 \( \mu \text{M} \), 189.6 \( \mu \text{M} \) and 270.3 \( \mu \text{M} \), respectively. As shown in Fig 1F, G, IC50 of SM was lower than that of TMZ group, and SM treatment didn’t affect the growth of normal neurons at anti-glioma dose. Our data suggest SM is a promising drug for the treatment of glioma.

**SM induces U87 cells apoptosis in vitro**

As shown in Fig 2, the early apoptosis rates of U87 cells (baseline: 1.25%) following SM (50 and 100 \( \mu \text{M} \)) treatment were 1.36% and 2.35% \( (P < 0.01) \), respectively. The late apoptosis rates of U87 cells (baseline: 3.98%) in response to SM were 6.14% and 16.95%, respectively. Our data demonstrate SM treatment significantly promotes apoptosis of U87 cells.

**SM promotes caspases activation and decreases anti-apoptotic proteins expression**

To study effects of SM on caspases activities in U87 cells, caspase-3, caspase-8 and caspase-9 activities were measured. Fig. 3A1–A3 indicate caspase-3, caspase-8 and caspase-9 activities were increased following SM treatment compared with that of control \( (P < 0.01) \). In addition, western blot analysis revealed SM reduced expression levels of anti-apoptotic proteins, including HIAP, Bcl-2 and survivin \( (P < 0.01, \text{Fig. } 3B1–B3) \).

**SM exhibits anti-glioma activity in vivo**

As shown in Fig. 4A, B, SM treated mice had lower tumour burden compared with that of control mice \( (P < 0.01) \). Strikingly, SM therapy remarkably prolonged survival time of the tumour mice \( (P < 0.01, \text{Fig. } 4C) \). No difference in the body weight was observed after SM treatment compared with control. However, TMZ therapy caused a slight decrease in the weight at day 18 after treatment \( (P < 0.01, \text{Fig. } 4D) \). These findings suggest SM was well tolerated by the recipient mice at therapeutic dose, and no

![Fig. 5. SM inhibits phosphorylation of STAT3 in U87 cells and U87 cells-bearing mice. A, A1: relative expression ratios of p-STAT3 and the protein bands of STAT3 and p-STAT3; B, B1: representative pictures of p-STAT3 positive cells and its ratios in tumour tissues; B2–B5: expression of HIAP, Bcl-2 and survivin in tumour tissues. The data were shown as mean ± SD, \( n = 3–5 \); **\( P < 0.01 \), compared to the control group.](image-url)
significant cytotoxicity was accompanied. Of note, SM and TMZ exhibit synergistic anti-tumour effects in vivo, as indicated by decreased tumour sizes and prolonged survival time compared with that of SM or TMZ group alone (P < 0.01, Fig. 4A–4C).

SM reduces p-STAT3 and anti-apoptotic proteins expression

As shown in Fig. 5A, a decreased expression of p-STAT3 was observed in U87 cells treated with SM compared with that of control cells (P < 0.01). However, the levels of STAT3 were unaffected in response to SM treatment. Additionally, we found SM significantly decreased the ratio of p-STAT3 positive cells (Fig. 5B, P < 0.01), and reduced expression of HIAP, Bcl-2 and survivin in tumour tissues (Fig. 5B2–B5, P < 0.01).

STAT3 mediates SM induced growth inhibition and apoptosis in U87 cells

IL-6 is an activator of STAT3 that induces oncogenesis (Banerjee and Resat, 2016; Nie et al., 2015). To disclose the role of STAT3 in SM induced growth inhibition, U87 cells were treated with SM in the presence or absence of IL-6 for 48 h. As shown in Fig. 6A, B, treatment of U87 with SM resulted in a significant decrease in the growth and increase in the apoptosis (P < 0.01), which was inhibited in presence of IL-6 (P < 0.01). Moreover, SM decreased the expression of p-STAT3 in U87 cells, and this effect was partly abolished by IL-6 stimulation (Fig. 6C, P < 0.01). Therefore, our results suggest STAT3 mediates SM induced growth inhibition and apoptosis in glioma cells.

Discussion

In the present study, we firstly studied effects of SM on glioma cells (mainly focused on U87) in vitro and in vivo. Our data indicate SM inhibited the growth of several glioma cell lines in vitro as well as U87 growth in the nude mice. These results are similar with previously published data performed in MDA-MB-231, MCF-7 and MCF-10A (Li et al., 2014). It should be noted that IC50 of SM were lower than that of TMZ, and SM prolonged survival time of mice more than TMZ. Moreover, SM with anti-glioma dose had little effect on the growth of normal cells and body weight, which was different with TMZ. Therefore, our data indicate the application of SM in clinic patients may be superior to that of TMZ.

An unexpected finding of current study was the synergic anti-glioma effect of SM and TMZ. In previous investigations (Liao et al., 2013; Wang et al., 2013; Zhang et al., 2014), SM synergistically inhibited esophageal carcinoma, colon carcinoma and gastric cancer cells growth with 5-fluorouracil. The possible reasons for synergic anti-tumour effects of SM and TMZ are: firstly, the anti-glioma mechanism of SM is different than that of TMZ. TMZ is an alkylating agent which breaks the DNA double-strand, thus causing cell cycle arrest and ultimately cell death, while SM induces cell autophagic death (Jiang et al., 2015) and promotes apoptotic death via modulating several apoptotic signalling pathways and proteins, including PI3K/Akt, MAPKs (Li et al., 2011) and cyclooxygenase-2 expression (Lv et al., 2011). Secondly, SM sensitizes multidrug-resistant colon cancer cells to doxorubicin by down-regulation of MDR-1 expression (Lv et al., 2011). TMZ resistance in glioblastoma is caused by MDR-1 over expression (Perazzoli et al., 2015). Our finding provides a new
therapeutic regimen by which SM could be used with TMZ together in the therapy of malignant glioma.

Induction of apoptosis in tumour cells is an important way to control malicious proliferation. Death receptor and mitochondrial pathways (Jin and El-Deiry, 2005) are two major pathways responsible for triggering apoptosis, which is featured by activation of caspase-8 and caspase-9, respectively. The two pathways converge at activation of caspase-3, an executioner of apoptosis (Jin and El-Deiry, 2005). In the present study, our data showed SM induced apoptosis of U87 cells, and increased activities of caspase-3, caspase-8 and caspase-9, which is consistent with previous reports conducted in lung cancer, colon carcinoma and hepatocellular carcinoma cells (Jiang et al., 2010; Lu et al., 2013; Zhang et al., 2014). These results suggest SM inhibited glioma cells proliferation by inducing apoptosis.

STAT plays important roles in modulating cell proliferation, differentiation, angiogenesis and immune response (Banerjee and Resat, 2016; Khanna et al., 2015). STAT3 is a member of STAT family. Phosphorylation of STAT3 leads to homodimerization, translocation to the nucleus and induction of target genes transcription, resulting in increased expression of oncogenesis related proteins, such as HIAP-1, Bcl-2 and survivin (Banerjee and Resat, 2016; Khanna et al., 2015; Nie et al., 2015). STAT3 has been confirmed to be overactive in glioma cells and the clinical patients (Hong et al., 2016; Rodrigues et al., 2016; Wang et al., 2016). In the present report, SM reduced p-STAT3 expression in U87 cells and tumour xenograft mice with decreased levels of HIAP-1, Bcl-2 and survivin. Interestingly, SM induced increase in apoptosis could be reversed by IL-6 interference. These outcomes suggest that SM exerts its anti-glioma effect by impairing activation of p-STAT3.

Conclusion

Taken together, our study firstly demonstrates SM effectively inhibits the growth of glioma cells in vivo and in vitro. The inhibitory effect of SM on glioma cells growth is via suppressing phosphorylation of STAT3. Our findings suggest SM is a promising therapeutic agent for glioma, and demonstrate inhibition of p-STAT3 is an efficient strategy for glioma prevention.

Conflict of interests

The authors state no conflicts of interest.

References


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