

Wogonin affects proliferation and the energy metabolism of SGC-7901 and A549 cells

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Abstract. Many studies have focused on the identification of therapeutic targets for the treatment of certain types of cancer. Wogonin is a natural flavonoid compound that exhibits a potent anti-cancer effect. The underlying mechanism of wogonin may therefore reveal an effective way to identify novel therapeutic targets. In the current study, growth curves and MTT assays were performed to determine the effects of wogonin in human gastric cancer cells (SGC-7901) and human lung adenocarcinoma cells (A549), respectively. Changes in morphology were observed using hematoxylin and eosin (H&E) staining. The activities of key enzymes in the glycolysis and tricarboxylic acid cycle were measured using spectrophotometry. Western blot analysis was performed to determine the expression levels of hypoxia inducible factor-1 α (HIF-1 α) and monocarboxylate transporter-4 (MCT-4). Wogonin inhibited cell proliferation in a time- and dose-dependent manner in SGC-7901 and A549 cells. H&E staining suggested that wogonin induced cell morphology changes. In SGC-7901 cells, lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH) activities and adenosine triphosphate (ATP) generation were decreased significantly by wogonin treatment compared with the untreated control. In A549 cells, wogonin significantly reduced LDH activity, but exhibited no significant effects on kinase activities or ATP generation. Furthermore, wogonin significantly decreased HIF-1 α and MCT-4 protein expression in SGC-7901 cells, but not in A549 cells. The results demonstrated that wogonin inhibited the energy metabolism, cell proliferation and angiogenesis in SGC-7901 and A549 cells by negatively regulating HIF-1 α and MCT-4 expression. The differential regulatory roles of wogonin in metabolism-associated enzymes in human gastric cancer and lung adenocarcinoma cells indicated its various antitumor mechanisms. The different metabolic regulatory

mechanisms exhibited by wogonin in different tumor tissues should therefore be considered for antitumor therapy.

Introduction

Wogonin is a flavonoid compound first isolated from *Scutellaria baicalensis* and is used in Chinese herbal medicine (1). It has been recognized as a potent anticancer agent due to its broad toxicity in various types of cancer cell lines, including human breast cancer, liver cancer, lung cancer and human gastric cancer cells (2-5). The underlying mechanisms of the growth-suppressive effects of wogonin on tumor cells are considered to be associated with inhibition of cell proliferation (6), induction of apoptosis (7), antiangiogenesis (8-12) and promotive effects on tumor cell differentiation (13). In addition, wogonin further exhibited pharmacologic properties, including neuroprotective, antiviral, anti-inflammatory and antioxyradical effects (14-16). Previously, various studies focused on exploring the underlying cellular pathways responsible for the energy metabolism in tumorigenesis. Increased catabolic glucose metabolism is one of the primary metabolic changes observed in proliferating cells (17). The shift in energy production in tumor cells from oxidative phosphorylation to glycolysis, regardless of the oxygen concentration, is a phenomenon termed 'Warburg effect' (18). Although the mechanisms and benefits of this metabolic behavior in tumor cells remain unclear, disturbance of the glycolysis emerges as a promising strategy for cancer therapy (19,20). The effects of wogonin on antiproliferative and apoptotic activities have been documented using various human cancer cells; however, its effects on energy metabolism-associated enzymes and adenosine triphosphate (ATP) generation in SGC-7901 and A549, human gastric cancer and human lung adenocarcinoma cell lines, respectively, remains to be elucidated.

Tumor cells have a unique aerobic glycolysis. Abnormal changes in glucose metabolism may exist in tumor cells and even in the presence of oxygen, glucose metabolism is transformed from oxidative phosphorylation to glycolysis, which consumes large quantities of glucose and generates lactic acid (21). In line with these characteristics, the present study attempted to evaluate different effects of wogonin on proliferation inhibition of SGC-7901 and A549 cells and further explored the sensitivity of these cell lines to wogonin, based on changes observed for various enzymes involved in the energy metabolism. The results suggested that in SGC-7901

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cells, wogonin inhibited the growth of tumor cells by interfering with the energy metabolism. Furthermore, decreased hypoxia inducible factor-1 α (HIF-1 α) and monocarboxylate transporter-4 (MCT-4) expression induced by wogonin may be partially responsible for inhibitory effects in the tumor metabolism. In A549 cells, wogonin demonstrated little influence on the energy metabolism. Since sensitivity to wogonin may be not the same in certain types of tumor cell, different anti-tumor therapy should therefore be considered when wogonin is used alone or in combination. The present study aimed to provide a guide for further studies on targeted therapy for different tumors types.

Materials and methods

Reagents and antibodies. Wogonin (Chengdu Institute of Biology, Chinese Academy of Science, Chengdu, China) was dissolved in dimethyl sulfoxide (DMSO; 100 mg/ml) and stored at -20°C. The solution was diluted as required using RPMI-1640 medium. 5-Fluorouracil (5-Fu) and MTT were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). SGC-7901 and A549 cell lines were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium, Fetal Bovine Serum (cat. no. 16000-044) and trypsin-EDTA 0.25% (cat. no. 25200-072) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Bicinchoninic acid (BCA) Protein Assay kit (cat. no. P0010), RIPA Lysis Buffer (cat. no. P0013B) and Trypan blue Staining Cell Viability Assay kit (cat. no. C0011) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Hexokinase (HK) assay kit (cat. no. A007-1), pyruvate kinase (PK) assay kit (cat. no. A076-1), lactate dehydrogenase (LDH) assay kit (cat. no. A020-1) and succinate dehydrogenase (SDH) activity assay kit (cat. no. A022). ATP assay kit (cat. no. A059-1) and Hematoxylin-Eosin stain kit (cat. no. D006) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Rabbit HIF-1 α (cat. no. PB0245) and MCT-4 (cat. no. PB0269) antibodies were obtained from Boster Biological Technology (Pleasanton, CA, USA). Mouse β -actin (cat. no. 60008-1), horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. SA00001-2) and goat anti-mouse IgG (cat. no. SA00001-1) antibodies were obtained from ProteinTech Group, Inc. (Chicago, IL, USA). The diaminobenzidine (DAB) kit (cat. no. ZLI-9018) was obtained from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (OriGene Technologies, Inc., Beijing, China). The enhanced chemiluminescence (ECL) kit (cat. no. 32109) was obtained from Thermo Fisher Scientific, Inc.

Measurements of cell growth and viability. SGC-7901 and A549 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator in an atmosphere of 5% CO₂ at 37°C as previously described (22,23). Cells were seeded in 96-well plates at a density of 5x10⁴ cells/well and incubated with 5% CO₂ at 37°C for 24 h. Subsequently, the cells were treated with wogonin at varying concentrations (5, 10, 15, 20, 25 and 30 μ g/ml) at 37°C for 48 h. An untreated control experiment was also performed. Cell viability was assessed by adding

MTT in PBS to a final concentration of 5 mg/ml. Plates were incubated at 37°C for a further 4 h. DMSO (150 μ l) was added to each well and incubated for 10 min at room temperature. The amount of MTT-formazan was directly proportional to the number of living cells and was determined by measuring the optical density at 490 nm. Cell survival rate was calculated using the following equation: Cell survival rate (%) = (Average OD_{wogonin} - Average OD_{control}) / (Average OD_{control} - Average OD_{blank}) x 100%. IC₅₀ were calculated using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Each experiment was repeated 4 times.

Trypan blue exclusion assay. SGC-7901 and A549 cells were seeded into 24-well plates at a density of 5x10⁴ cells/well and incubated with 5% CO₂ at 37°C for 24 h. Following pretreatment with wogonin (15 μ g/ml) or 5-Fu (10 μ g/ml) at 37°C for 24 h, a cell suspension was prepared at a suitable dilution (1.0x10⁵ cells/ml) in PBS. A total of 50 μ l of cell suspension was taken and mixed with an equal volume of 0.4% Trypan blue. The number of viable cells was counted under a light microscope at a magnification of x20 once daily for 7 days, and the growth curve was constructed. An untreated control group was analyzed additionally. Each experiment was repeated 4 times and the results were expressed as the mean \pm standard deviation for each treatment group.

Cell morphological assessment. SGC-7901 and A549 cells were seeded at a density of 5x10⁴ cells/well on coverslips, which were placed in 6-well plates and incubated with 5% CO₂ at 37°C for 24 h. Subsequently, the cells were treated with wogonin (15 μ g/ml) at 37°C for 48 h. An untreated control experiment was performed in parallel. Cells were washed with ice-cold PBS and fixed with 95% ethanol for 15 min at room temperature, followed by staining with hematoxylin (5 mg/ml) for 5 min and eosin (5 mg/ml) for 3 min at room temperature. Then the coverslips were then dehydrated with graded ethanol for 1-2 sec at room temperature and soaked in xylene for 5 min, mounted at room temperature and dried at 37°C for 24 h. Morphological changes of the cells were observed under a light microscope at a magnification of x40. Furthermore, the number of cells in each group were counted at a magnification of x20 and evaluated. Each experiment was repeated 4 times.

Enzyme activity assays. After SGC-7901 and A549 cells were seeded into 6-well plates at a density of 5x10⁵ cells/well, cells were pretreated with wogonin (15 μ g/ml) for 48 h at 37°C. An untreated control experiment was also performed. Following pretreatment, cells were trypsinized with Trypsin-EDTA and suspended in 0.9% physiological saline, then centrifuged at 2,000 x g for 5 min at 4°C. HK, PK, LDH and SDH activities were assessed with activity assay kits by measuring the absorbance at 340, 440 and 600 nm, respectively. Enzyme activities were normalized against the protein concentration, determined using the BCA Protein Assay kit at 562 nm. Each experiment was repeated 4 times.

Western blot analysis. HIF-1 α and MCT-4 expression were examined by western blot analysis. After SGC-7901 and A549 cells reached a confluence of 70%, they were incubated with wogonin (15 μ g/ml) for 48 h with 5% CO₂ at 37°C in cell culture

flasks. Cells were then washed with ice-cold PBS 3 times and the total protein was extracted with RIPA Lysis Buffer. Samples were then centrifuged at 13,500 x g for 15 min at 4°C to remove cell debris. An untreated control experiment was also performed. The protein concentration was measured using the BCA Protein Assay kit. Protein (~30 µg) was loaded and separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Following 1 h of blocking with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) at room temperature, the membranes were incubated with primary antibodies with gentle shaking overnight at 4°C; HIF-1α (1:100), MCT-4 (1:100) and β-actin (1:1,000) prepared in 5% non-fat dried milk in TBS-T. Membranes were further incubated with secondary antibodies for 1 h at room temperature, then developed using an ECL kit and scanned densitometry. The band densities were quantified via densitometry using Quantity One Software 4.4 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The intensities of the protein bands were normalized against β-actin. Each experiment was repeated 4 times.

Statistical analysis. Data are expressed as mean ± standard deviation. Statistical analysis was performed with SPSS (version 20.0; IBM Corp., Armonk, NY, USA). Differences between the conditions tested were identified using one-way analysis of variance and differences among groups were analyzed for significance using a Tukey's test. Comparisons between two groups were performed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Wogonin affects the proliferation of SGC-7901 and A549 cells. To evaluate the effect of wogonin on cell proliferation, SGC-7901 and A549 cells were treated with varying concentrations of wogonin and viability was evaluated by MTT assays. As presented in Fig. 1A, the results suggested that the inhibition rates of wogonin in A549 cells were stable at high concentrations (10-30 µg/ml). Measured rates were all similar to the IC₅₀ (18.17 µg/ml) for A549 cells. To achieve high efficiency and low toxicity, 15 µg/ml wogonin were used in subsequent experiments with A549 cells. In SGC-7901 cells, the inhibitory effect of wogonin on cell proliferation was dose-dependent between 5 and 20 µg/ml. Although the inhibitory rate was higher at 20 µg/ml compared with the 15 µg/ml treatment, to achieve lower toxicity and to be consistent with the A549 cells, 15 µg/ml wogonin in SGC-7901 cells was selected for further research. The inhibitory rates of SGC-7901 and A549 cells following wogonin treatment (15 µg/ml) were 35.00±0.12 and 54.17±0.24%, respectively, indicating a stronger inhibitory effect of wogonin on A549 cell proliferation compared with SGC-7901 cells.

To investigate whether the effect of wogonin on cell proliferation was time-dependent, SGC-7901 and A549 cells were treated with 15 µg/ml wogonin for 7 days and the cell numbers were counted daily. Growth curves were prepared to describe the effect of wogonin on cell proliferation over time. As presented in Fig. 1B, wogonin decreased the cell proliferation in the cell lines in a time-dependent manner compared

with the untreated control. Treatment with wogonin markedly decreased cell number in a time-dependent manner in each cell line, which was similar in strength to the effect exhibited by 10 µg/ml 5-Fluorouracil (5-Fu). 5-Fu is a potent anti-tumor agent that affects pyrimidine synthesis by inhibiting thymidylate synthetase, thus depleting intracellular dTTP pools (24). The results suggested that wogonin significantly inhibited the proliferation of SGC-7901 and A549 cells over time compared with an untreated control.

Cell morphological assessment following wogonin treatment. Morphological changes induced by Wogonin (15 µg/ml) were observed by H&E staining. As presented in Fig. 2, the majority of treated cells exhibited a round in shape, loss of cell volume, hyperchromatic nuclei and cytoplasm agglutination compared with the untreated controls (Fig. 2A-D). Compared with controls, the cell numbers were significantly decreased following treatment with wogonin in SGC-7901 and A549 cells (Fig. 2E and F). These results indicated that wogonin may induce cell morphological changes in the studied cell lines.

Wogonin affects ATP generation and the activities of energy metabolism-associated enzymes. To investigate the effects of wogonin on energy metabolism, activities of enzymes participating in glucose anaerobic glycolysis, including HK, PK and LDH, and the activity of SDH, a contributor to the tricarboxylic acid cycle (TAC), were measured. As presented in Table I, SDH activity and ATP levels were significantly decreased by wogonin in SGC-7901 cells when compared with the untreated control (P<0.05). The inhibitory effect of wogonin significantly impacted on LDH activity when compared to the untreated control (P<0.01). Compared with the controls, A549 cells treated with wogonin significantly inhibited LDH activity (P<0.01) and ATP content was decreased (P<0.05). HK, SDH and PK activities were not significantly affected. The results indicated that wogonin may affect lactic acid and cell energy generation in SGC-7901 cells. Furthermore, wogonin treatment only regulated lactic acid and ATP generation in A549 cells.

Wogonin affects HIF-1α and MCT-4 expressions. To further investigate the effect of wogonin on energy metabolism-associated enzymes, HIF-1α and MCT-4 expression were determined by western blot. As presented in Fig. 3, following treatment with wogonin for 48 h, HIF-1α and MCT-4 expression significantly decreased in SGC-7901 cells compared with the control (P<0.01; Fig. 3A), but no significant changes were observed in A549 cells (Fig. 3B).

Discussion

Scutellaria has been used in Chinese herbal medicine for the treatment of inflammation, cancer, allergies and bacterial and viral infections of the respiratory and gastrointestinal tract (25). Wogonin is one of the effective compounds extracted from *Scutellaria*, exhibiting multiple pharmacological effects, including cytotoxic effects in human cancer cell lines, inhibitory effects on tumor angiogenesis and neoplasm metastasis (26).

The present study performed MTT assays to measure the inhibitory rates of wogonin in several cancer cell lines and it

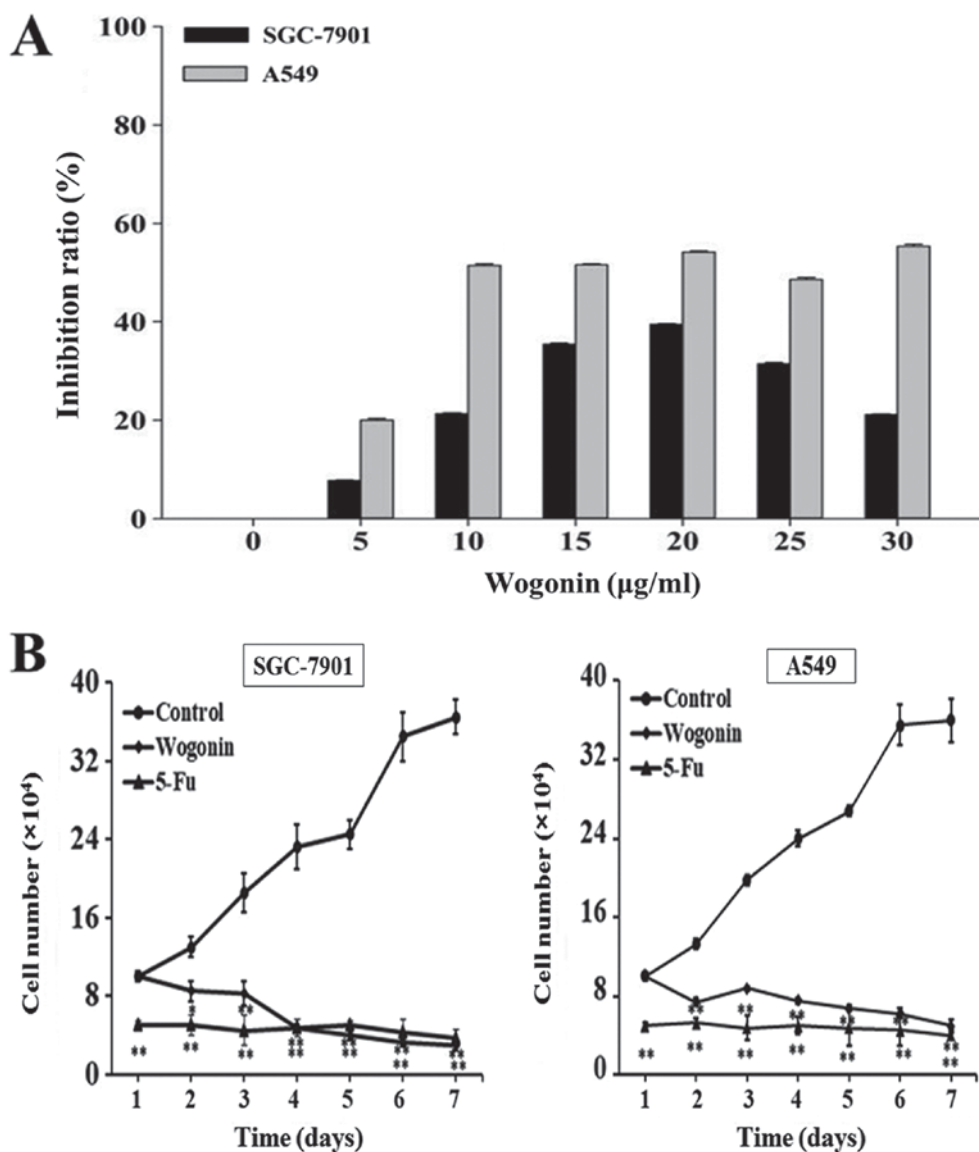


Figure 1. Effects of wogonin on cell proliferation. (A) Wogonin inhibits the proliferation rate of SGC-7901 and A549 cells. Inhibition ratios for varying amounts of wogonin were determined by MTT assay following treatment for 48 h. (B) Growth curves of SGC-7901 and A549 cells. Cells were treated with 15 µg/ml wogonin and monitored over 7 days. *P<0.05 and **P<0.01 vs. control; n=4.

was demonstrated that human gastric cancer cells and human lung adenocarcinoma cells were sensitive to wogonin treatment. The inhibitory effects of wogonin on cell proliferation were evaluated and the maximal inhibitory rate was induced by 15-20 µg/ml wogonin, suggesting a high inhibitory efficiency on cell proliferation. Furthermore, according to the inhibitory rates exerted by wogonin (15 µg/ml), a stronger effect on A549 cell proliferation was observed when compared with SGC-7901 cells, thus it is necessary and essential to launch a contrastive study on the effects of wogonin in these two cell lines. Growth curves and H&E staining results suggested that, wogonin (15 µg/ml) may inhibit the cell proliferation in a time-dependent manner. 5-Fu is a first-line adjuvant chemotherapeutic agent that is often administered as part of a regimen with other cytotoxic drugs, including Cisplatin. It is also a potent antitumor agent that affects pyrimidine synthesis by inhibiting thymidylate synthetase (27,28). In the current study, 5-Fu was selected as a positive control to further assess

the inhibitory effects of wogonin on SGC-7901 and A549 cell proliferation. The observed effects on proliferation induced by wogonin were not significantly different compared with the effects exhibited by 5-Fu treatment.

Cancer is considered a metabolic disease, which requires a lot of energy for proliferation, metastasis, infiltration and other physiological functions (29). Even in the presence of ample oxygen, cancer cells preferentially metabolize glucose by glycolysis, which is a less efficient pathway for producing ATP when compared with oxidative phosphorylation (30). In highly proliferating tumors, blood supply may become insufficient and cancer cells are exposed to hypoxia, which may upregulate HIF-1, which in turn mediates overexpression of glycolytic enzymes and the upregulation of glucose transporters (GLUT) (31,32). In addition, HIF stimulates angiogenesis by upregulating several factors, including the vascular endothelial growth factor, GLUT-1 and LDH (33-35). In tumor cells, glucose is metabolized to lactate and the

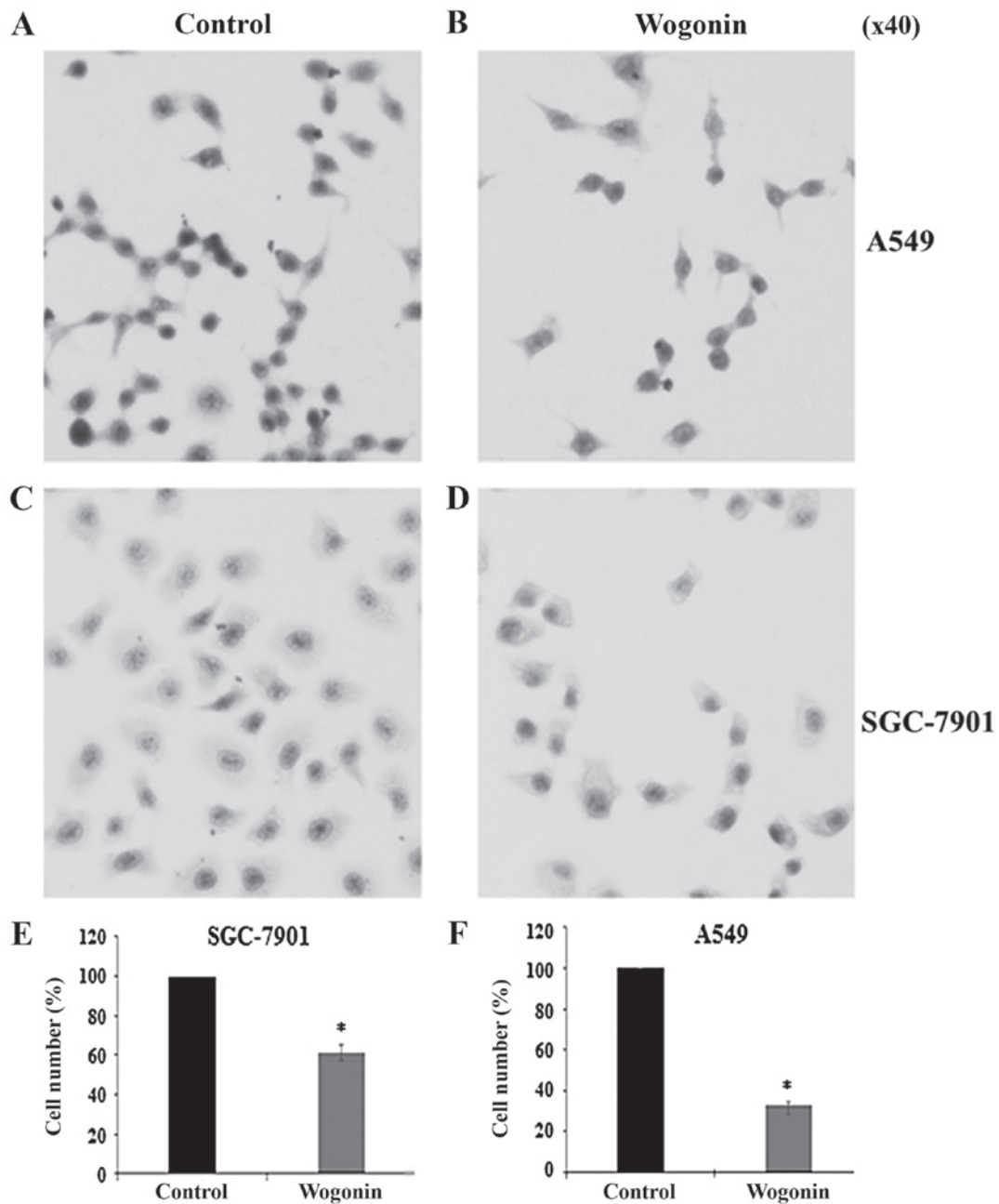


Figure 2. H&E staining of A549 and SGC-7901 cells. H&E staining of (A) untreated A549 cells and (B) A549 treated with wogonin (15 μ g/ml; magnification, x40). H&E staining of (C) untreated SGC-7901 cells and (D) SGC-7901 treated with wogonin (15 μ g/ml; magnification, x40). Cell numbers in the control and wogonin-treated group for (E) A549 and (F) SGC-7901 cells, with the cell number in control group set as 100%. *P<0.05 vs. control; n=4. H&E, hematoxylin and eosin.

latter is exported from cells by MCT-4, which results in an accumulation of lactate, lowering the pH in the tumor microenvironment (36,37). Tumor cells primarily perform glycolysis instead of oxidative phosphorylation and it is well known that this metabolic alteration is important for tumor development and progression and is a hallmark of cancer (38). HK and PK are two rate-limiting enzymes in the glycolysis, which catalyze the initial step of glycolysis and the dephosphorylation of phosphoenolpyruvate to pyruvate, respectively (39). LDH is a glycolytic enzyme, which catalyzes the reversible conversion of lactate to pyruvic acid, therefore contributing to the acidic microenvironment (40). SDH participates in the TAC and is located on the inner mitochondrial membrane (41). Functions

of mammalian SDH extent from mitochondrial energy generation to oxygen sensing and tumor suppression (42).

In the current study, activities of three glycolytic enzymes, HK, PK and LDH, and SDH, HIF-1 α and MCT-4 expression, and ATP levels were measured in SGC-7901 and A549 cells treated with wogonin. Wogonin exhibited different effects in the two cell lines. In SGC-7901 cells, wogonin significantly decreased the activities of LDH and SDH, and the ATP level compared with an untreated control. In cancer progression, increasing glucose consumption leads to the accumulation of lactate, which lowers the pH in the tumor microenvironment (43). Wogonin may improve the acidity of the microenvironment by reducing lactate generation and

Table I. Energy metabolism-related enzymes activities and ATP level.

Enzyme activity	Wogonin (SGC-7901)	Control (SGC-7901)	Wogonin (A549)	Control (A549)
HK (U/g prot)	49.80±5.41	56.56±6.49	48.61±0.41	47.09±6.49
PK (U/g prot)	2,156.56±135.61	1,975.76±94.71	2,507.98±191.61	3,206.05±469.47
LDH (U/g prot)	216.22±8.95 ^b	3301.54±263.39	8406.95±506.35 ^b	12,805.51±803.25
SDH (U/mg prot)	239.07±6.83 ^a	382.46±15.89	525.27±46.54	511.90±36.24
ATP (μmol/g prot)	3,090.50±456.35 ^a	4,595.84±563.52	1,219.45±314.87 ^a	2,428.03±203.99

Values are expressed as the mean ± standard deviation, n=4. ^aP<0.05 and ^bP<0.01 vs. the control. HK, hexokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; SDH, succinate dehydrogenase.

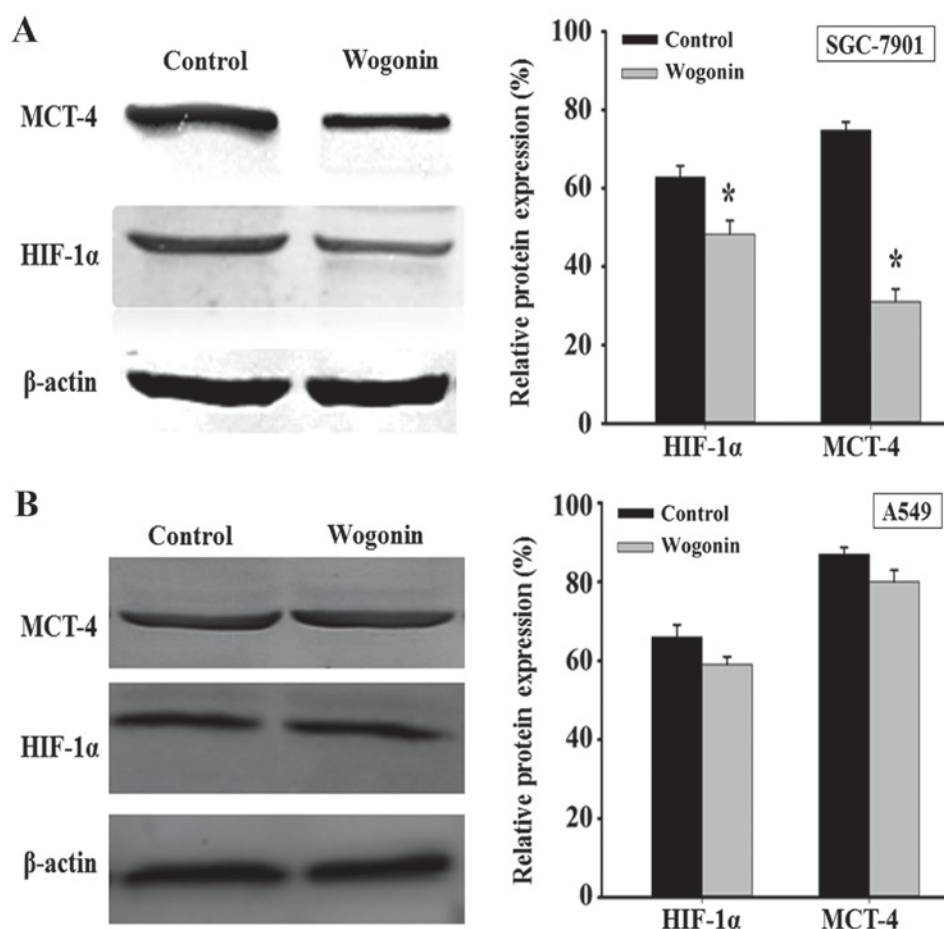


Figure 3. Effects of wogonin on HIF-1 α and MCT-4 expression. HIF-1 α and MCT-4 expression was determined in (A) SGC-7901 and (B) A549 cells treated with wogonin (15 μ g/ml) for 48 h. Expression levels of HIF-1 α and MCT-4 significantly decreased in SGC-7901 compared with the control, but not in A549. *P<0.05 vs. control; n=4. HIF-1 α , hypoxia inducible factor-1 α ; MCT-4, monocarboxylate transporter-4.

potentially affect glycolysis through the inhibition of different glycolytic enzymes. In comparison, wogonin inhibited the activity of LDH and the generation of ATP in A549 cells, and exhibited no significant effects on activity of the other enzymes. In summary, wogonin may decrease lactic acid and ATP generation in A549 cells, but may not affect the other elements to the energy generation process.

The current study evaluated effects of wogonin on various enzymes involved in glycolysis, but only one enzyme participated in the process of aerobic oxidation. Further key enzymes, including citrate synthase, isocitrate dehydrogenase

and α -oxoglutarate dehydrogenase, involved in TAC may be analyzed in future experiments to evaluate the effects of wogonin on aerobic oxidation.

In addition, it has been suggested that the phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K) signaling pathway serves a critical role in cancer metabolism and progression (44). The PI3K signaling pathway regulates glucose uptake via protein kinase B by regulating GLUT-1 expression, enhancing glucose capture and stimulating phosphofructokinase activity, this may cause a dependency of the cells on high levels of glucose (45). Intracellular mammalian

target of rapamycin, which is an upstream mediator of HIF-1 activation, is further activated via the PI3K signaling pathway (46). HIF-1 activation upregulates GLUTs and HK expression in tumor cells (47). The pathways through which wogonin affected the energy metabolisms in the current study require further verification. Furthermore, in order to demonstrate the antitumor activity of wogonin and to evaluate the role wogonin serves in the energy metabolisms, tumor animal models may be established for *in vivo* experiments.

In summary, the findings of the present study indicated that wogonin may affect the energy metabolism and the acidic microenvironment in SGC-7901 cells by decreasing HIF-1 α and MCT-4 expressions. In A549 cells, wogonin exhibited no significant effects on the energy metabolism, indicating that the strong inhibitory effect of wogonin on cells proliferation may be induced by other mechanisms, but not by the inhibition of the energy metabolism. The growth of cells cannot continue without the supply of energy. While inhibiting cell proliferation, wogonin interferes with changes in certain proteins and key enzymes during cellular energy metabolism. The current study hypothesizes that the combination of wogonin and certain enzyme inhibitors in energy metabolism may prevent the supply of energy to tumor cells and therefore inhibit tumor cell proliferation.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SJW and JNZ conceived and designed the experiments of the current study. JNZ, JKZ, SR, WWS and WJZ performed the experiments. JNZ, WWS, JKZ, SR and WJZ contributed reagents/materials/analysis tools. JNZ wrote the manuscript and SW revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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