

Original Research Article

Wogonoside exerts potential anti-tumor activity against bladder cancer *in vivo* and *in vitro* via regulation of GSK-3 β /ERK/AKT signaling pathway

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Abstract

Purpose: To explore the antitumor activity of wogonoside on bladder cancer, and its underlying mechanism of action.

Methods: Methyl thiazolyl tetrazolium (MTT) assay was applied to determine the anti-proliferative activity of wogonoside (2 - 128 μ M) on bladder cancer 5637 cell line at various times, and the half-maximal inhibitory concentration (IC_{50}) was measured. The antitumor activity of wogonoside (30 mg/kg, ip) against bladder cancer 5637 cell line was evaluated in nude mice bearing human bladder cancer 5637 cells. Additionally, western blotting and enzyme-linked immunosorbent assay (ELISA) were carried out to investigate the levels of the caspase-3, caspase-9, B cell lymphoma/leukemia-2 (Bcl-2), Bcl-2 associated X-protein (Bax), phosphorylated (p)-glycogen synthase kinase (GSK)-3 β , p-extracellular signal-regulated kinases (p-ERK), and p-(protein kinase B) AKT.

Results: The *in vitro* results revealed that wogonoside exerted anti-proliferative activity against bladder cancer 5637 cells with an IC_{50} of 20.59 μ M ($p < 0.01$), in a concentration- and time-dependent manner. Furthermore, wogonoside treatment also significantly suppressed tumor volume in mice ($p < 0.01$). The potential mechanisms were mainly associated with apoptosis mediated by mitochondria via up-regulation of caspase-3, caspase-9, and Bax levels and down-regulation of Bcl-2, p-GSK-3 β , p-ERK, and p-AKT.

Conclusion: The results reveal that wogonoside has remarkable anti-tumor potentials against bladder cancer. Further translational studies are warranted to test the clinical application of this medicinal agent in bladder cancer.

Keywords: Bladder cancer, Wogonoside, GSK-3 β /ERK/AKT signaling pathway, Apoptosis, Cell line 5637

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INTRODUCTION

Bladder cancer, one of the most frequently occurring tumors in men, is also a leading cause

of cancer deaths among people [1-3]. Furthermore, epidemiological investigations have reported a low 5-year survival for bladder cancer. The recurrence rate of bladder cancer remains

extremely high, with up to 90 % reported in some studies [4-6]. Consequently, more novel therapeutic strategies and reliable active agents for treating bladder cancer are urgently needed. Previous investigations mainly focused on the potential ability of natural products derived from herbal plant to prevent or treat cancers. It is certain that natural products are important resource for finding novel and effective drugs with low toxicities [7,8].

Scutellaria baicalensis is a commonly used herbal medicine in China for treating various tumors and inflammatory diseases [9]. Wogonoside is one of the main active constituents isolated from *S. baicalensis*, which has been proven to possess numerous pharmacological activities by *in vivo* and *in vitro* studies. However, few reports have focused on the anti-tumor effect of wogonoside.

Therefore, we aimed to explore the anti-tumor activity of wogonoside against bladder cancer and the potential molecular mechanisms mediating this effect.

EXPERIMENTAL

Chemicals and reagents

Wogonoside (with purity greater than 98%) was obtained from Shanghai Tauto Biotech. Co. Ltd. (China, http://www.tautobiotech.com/products_gmlc.htm) Dulbecco's modified eagle's (DMEM) and fetal bovine serum (FBS) from Invitrogen Co. (Shanghai, China); methyl thiazolyl tetrazolium (MTT) from Sigma-Aldrich (Shanghai, China); enzyme-linked immunosorbent assay (ELISA) kits for caspase-3 and caspase-9, B cell lymphoma/leukemia-2 (Bcl-2) and Bcl-2 associated X protein (Bax), phosphorylated (p)-glycogen synthase kinase (GSK)-3 β , p-extracellular signal-regulated kinases (ERK1/2), p-protein kinase B (AKT), and GAPDH antibodies from Abcam Biotech (Cambridge, MA, USA); Radio-Immunoprecipitation Assay (RIPA) lysis buffer, BCA protein assay reagent, secondary antibodies, and enhanced chemiluminescence (ECL) kits from the Beyotime Biotech. (Shanghai, China).

Cell culture

Human bladder cancer 5637 cell line, applied to explore the anti-bladder tumor activity of wogonoside and the related molecular mechanisms in the current study, was acquired from American Culture Collection. The cell lines

were cultured in humidified atmosphere (37 °C, 5 % CO₂) with DMEM added 10 % FBS.

Cell viability assay

MTT assay, according to previous report, was carried out to evaluate the viability of cancer 5637 cells [10]. Cells were seeded into a 96-well plate at 1×10^5 cells/mL, which were then exposed to wogonoside at different concentrations of 0 (control), 2, 4, 8, 16, 32, 64, and 128 μ M for another 24 h. The microplate reader (Bio-rad, CA, USA) was employed to measure the absorbance at 570 nm, and the IC₅₀ value of wogonoside against 5637 cells was calculated. Furthermore, to investigate the anti-proliferative effect of wogonoside in time-dependent, 5637 cells were treated with wogonoside at 8, 16, and 32 μ M for 12, 24, 36, 48, and 72 h. The proliferation inhibition (%) of wogonoside against bladder cancer 5637 cells was detected as in equation (Eq) 1.

$$\text{Inhibition (\%)} = \{(Abc - Abw)/Abw\} \times 100 \dots\dots(1)$$

where *Abc* and *Abw* represented the absorbance values of control and wogonoside, respectively.

Assessment of anti-tumor effect of wogonoside *in vivo*

In this study, 12 nude mice totally were divided into control and wogonoside (30 mg/kg, i.p.) group (n = 6). Tumor-bearing mice were prepared according to the previous report with minor modification [4]. Briefly, bladder 5637 cancer cells were given into the right flank of mice at 2×10^6 cells/mouse by subcutaneous method. The bearing-tumors mice were subcutaneously treated with wogonoside at 30 mg/kg once the tumors grew to 2 - 3 mm in diameter and an equal volume of 0.5 % DMSO (i.p.) was given to the control mice. The tumor diameters of mice were determined using a Vernier caliper within 15 days (once 5 days), and the tumor volumes were calculated as follows: Tumor volume = (width² × length)/2 [11].

Determination of caspase-3 and caspase-9 activities in 5637 cells

The bladder cancer 5637 cells were seeded into a 6-well plate at 1×10^5 cells/mL. The 5637 cells were then exposed to wogonoside at concentrations of 8, 16, and 32 μ M. Then, the bladder cancer 5637 cells were collected for ELISA experiments to determine caspase-3, caspase-9 levels in 5637 cells according to manufactures' instructions.

Western blotting assays

Human bladder cancer 5637 cells (1×10^5 cells/mL) were seeded into 6-well plates for 24 h. Then, the cell lines or tumor tissues were collected, and the proteins were extracted using RIPA lysis buffer. After determination of protein concentrations by a BCA kit, equal amounts of 30 μ g proteins were parted with SDS-PAGE. The primary antibodies of Bcl-2 (1:1000), Bax (1:2000), p-GSK-3 β (1:1000), p-ERK1/2 (1:1000), and p-AKT (1:1000) were incubated, which were then incubated with the secondary antibodies. The specific bands were analyzed via ECL chemiluminescence kit. GAPDH was used as the internal reference for normalizing protein.

Statistical analysis

All the data are presented as mean \pm standard deviation (SD), and they were calculated using 19.0 SPSS software (Chicago, IL, USA) by one-way ANOVA. $P < 0.05$ was statistically regarded as significant.

RESULTS

Inhibitory activity of wogonoside on bladder cancer 5637 cells *in vitro*

The anti-tumor potential of wogonoside against 5637 cell lines *in vitro* was determined in the present study. As shown in Figure 1 A, wogonoside showed a significant anti-proliferative activity, in a dose-dependent manner at 2 - 128 μ M (Figure 1 A), against the tested cancer 5637 cell line with an IC_{50} value of 20.59 μ M. Additionally, the results shown in Figure 1 B indicated that wogonoside also exhibited prominent inhibitory effect against 5637 cell lines, in a time-dependent method within 72 h (Figure 1 B).

Pro-apoptotic effects of wogonoside on bladder cancer 5637 cells *in vitro*

As observed in the Figure 2, the present findings showed that wogonoside (8, 16, and 32 μ M) could concentration-dependently increase the levels of caspase-3 ($p < 0.01$), caspase-9 ($p < 0.01$), when compared to the control group. Furthermore, the effects of wogonoside on levels of Bcl-2 and Bax were evaluated by western blot (Figure 3). The results indicated that, compared to the control group, wogonoside at 8, 16, and 32 μ M gradually decreased the Bcl-2 level ($p < 0.01$) but obviously increased the Bax ($p < 0.01$). These findings reveal that the anti-proliferative effects of wogonoside on bladder cancer 5637

cells might associate with apoptosis-inducing effect.

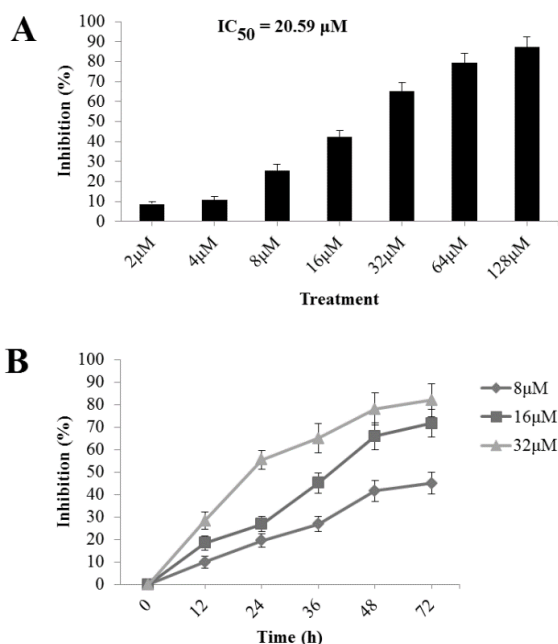


Figure 1: The anti-proliferative activity of wogonoside against the bladder cancer 5637 cell line. (A) The cancer 5637 cells were exposed to wogonoside at 2 - 128 μ M for 24 h. (B) The cancer 5637 cells were treated with wogonoside at 8, 16, and 32 μ M for 0, 12, 24, 36, 48, and 72 h. ($n = 4$)

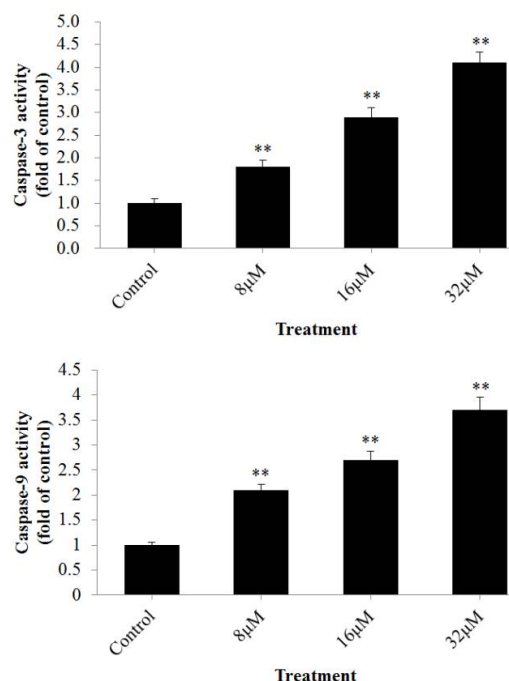


Figure 2: Effects of wogonoside (8, 16, and 32 μ M) on caspase-3 and caspase-9 expressions in 5637 cells. Data are represented as the mean \pm SD ($n = 4$); ** $p < 0.01$ vs. the control

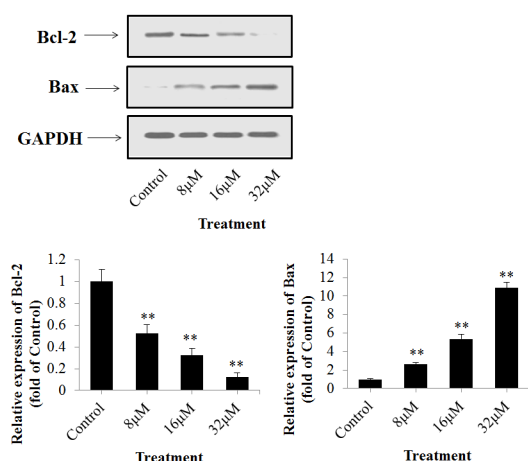


Figure 3: Effect of wogonoside (8, 16, and 32 µM) on the Bcl-2 and Bax expressions levels in 5637 cells. Data are represented as the mean ± SD (n = 4); **p < 0.01 vs. the control

Wogonoside down-regulated p-GSK-3β, p-ERK1/2, and p-AKT in 5637 cells

To investigate the underlying molecular mechanisms of antitumor activity of wogonoside on 5637 cells, western blotting assays were carried out to evaluate the protein expressions of p-GSK-3β, p-ERK1/2, and p-AKT in 5637 cells after treatment with wogonoside. As can be seen from Figure 4, following treatment with wogonoside at 8, 16, and 32 µM, protein expressions of p-GSK-3β, p-ERK1/2, and p-AKT in 5637 cells were dose-dependently down-regulated (p < 0.01).

Anti-tumor effect of wogonoside on tumor-bearing mice

The *in vitro* results indicated that wogonoside exerts an anti-proliferative effect on bladder cancer 5637 cell lines by induction of the mitochondrial pathway of apoptosis. Therefore, the anti-tumor effect of wogonoside against bladder cancer 5637 cells *in vivo* was further investigated by replanting tumor on nude mice model. As shown in Figure 5 A, wogonoside at 30 mg/kg obviously suppressed tumor growth within a 15-day observation period (p < 0.01). There were no evident differences in mice body weight between the control and wogonoside group, indicated as p > 0.05 (Figure 5 B). These findings reveal that wogonoside exhibits an anti-tumor potential on mice bearing tumors of 5637 cells *in vivo*. As presented in Figure 6, compared to the control mice, caspase-3 expression level was markedly increased in wogonoside-treated nude mice (p < 0.01), whereas p-GSK-3β, p-ERK1/2, and p-AKT (p < 0.01) were decreased.

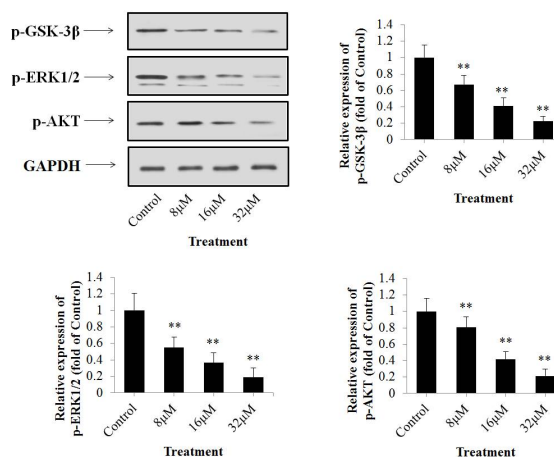


Figure 4: Effect of wogonoside (8, 16, and 32 µM) on the p-GSK-3β, p-ERK1/2, and p-AKT expression levels in 5637 cells. Data are represented as the mean ± SD (n = 4); **p < 0.01 vs. the control

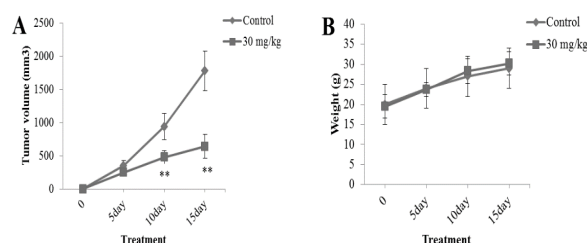


Figure 5: The anti-tumor activity of wogonoside (30 mg/kg) on nude mice bearing 5637 tumor cells. (A) Effects of wogonoside on tumor volumes and (B) body weights of 5637 tumor-bearing mice. Data are represented as the mean ± SD (n = 6); **p < 0.01 vs. the control

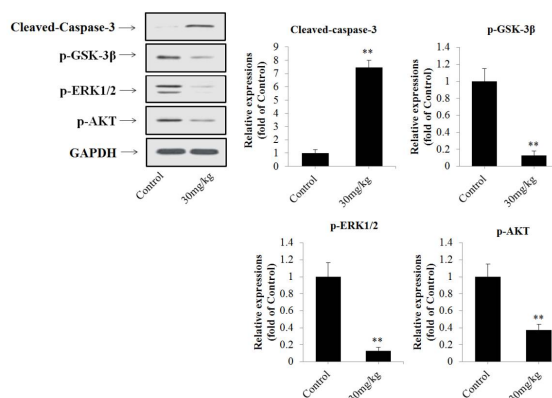


Figure 6: Effect of wogonoside (30 mg/kg) on the expression levels of caspase-3, p-GSK-3β, p-ERK1/2, and p-AKT in tumor tissues. Data are represented as the mean ± SD (n = 6); **p < 0.01 vs. the control

DISCUSSION

Bladder cancer is considered as the most serious cancer of the urinary system with an increasing morbidity and fatality [2]. Nowadays, the

available strategies for treating bladder cancer commonly employed synthetic chemotherapeutics, such as methotrexate, doxorubicin, and cisplatin. However, due to long-term use of these drugs, serious drug-resistances and toxicities have appeared [12]. The results in the present investigation revealed that wogonoside possesses significant antitumor effects against bladder cancer 5637 cells, and the possible underlying mechanisms are related to regulation of the p-GSK-3 β /p-ERK1/2/p-AKT signaling pathway.

Recently, it was reported that unregulated cell proliferation and apoptosis disruption would result in the development of cancers [13]. Apoptosis or programmed cell death, a physiological cell suicide process, is recognized as an effective strategy for cancer treatment [14]. In addition, apoptosis induced by mitochondria is an important apoptosis pathway. The caspase family proteins could be activated by regulating the cytochrome *c* releasing into the cytoplasm of Bcl-2 family proteins [15-17]. The proportion between the Bcl-2 protein and Bax plays an essential role in cytochrome *c* releasing into the cytoplasm and caspase proteins activation. Besides, caspase-3 activation is a crucial event for executing cell apoptosis, and the expression or activity of caspase-3 is a bio-marker for cells undergoing apoptosis [18,19]. The western blotting assay demonstrated that wogonoside could not only increase the expression level of caspase-3 and Bax, but could also decrease Bcl-2 in 5637 cells, indicating that the anti-proliferative effect of wogonoside against human bladder cancer 5637 cells may be mediated via the mitochondrial-mediated pathway of apoptosis.

The ERK1/2/MAPK signaling pathway acts as a critical role in the formation of bladder cancer. Abnormal activation of ERK1/2/MAPK would result in loss of cell apoptosis and malignant transformation of cancer cells [20,21]. Thus, it is generally considered that blocking of the ERK1/2/MAPK signaling pathway could be beneficial for the treatment of bladder cancers in clinic. It is also reported that AKT protein is significant for the PI3K/AKT signaling pathway, and inhibition of AKT phosphorylation is beneficial for treating a variety of cancers [22,23]. GSK-3 β is another crucial protein in the AKT signaling pathway, and suppression of GSK-3 β phosphorylation would decrease tumor cell proliferation but increase apoptosis of liver cancer cells. Wogonoside, isolated from *Scutellaria baicalensis*, significantly down-regulated ERK1/2, AKT, and GSK-3 β

phosphorylation levels in bladder cancer 5637 cells.

CONCLUSION

The findings reveal that wogonoside exerts potential anti-tumor effects against bladder cancer 5637 cells *in vivo* and *in vitro* via down-regulation of GSK-3 β /ERK/AKT signaling pathway. The results provide a new insight into the pharmacological role of wogonoside in the clinical management of bladder cancer.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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