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Original Research Article

Wogonoside exerts potential anti-tumor activity against bladder cancer *in vivo* and *in vitro* via regulation of GSK-3β/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 evaluate the anti-proliferative activity of wogonoside (2 - 128 μM) against bladder cancer 5637 cell line at different times, and the half maximal inhibitory concentration (IC_{50}) was measured. The antitumor activity of wogonoside (30 mg/kg, i.p.) against bladder cancer 5637 cell line was confirmed via in vivo experiments on nude mice bearing human bladder cancer 5637 cells. Additionally, western blotting assay and enzyme-linked immunosorbent assay (ELISA) were used to investigate expression levels of caspase-3, caspase-9, B cell lymphoma/leukemia-2 (Bcl-2), Bcl-2 associated X protein (Bax), phosphorylated (p)-glycogen synthase kinase (GSK)-3β, p-extracellular signal-regulated kinases (p-ERK), and p-(protein kinase B) AKT.*
Results: *The in vitro results reveal that wogonoside has remarkable anti-proliferative activity against bladder cancer 5637 cells with IC_{50} of 20.59 μM, in a concentration-and time-dependent manner. Furthermore, wogonoside treatment also suppressed tumor volume of nude mice bearing bladder cancer 5637 cell ($p < 0.01$). The potential mechanisms seems to be 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-3β, p-ERK, and p-AKT.*
Conclusion: *The results reveal that wogonoside possesses 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-3β/ERK/AKT signaling pathway, Apoptosis, 5637 Cell line*

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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 reported that patients with bladder cancer have a low survival time of 5 years and extremely high

recurrence rate, with up to 90 % reported in some studies [4-6]. It is certain that natural products derived from herbal plant are important resource for preventing or treating bladder cancers with low toxicities and side-effects [7,8].

Scutellaria baicalensis is a commonly used herbal medicine in China for treating various tumors and inflammatory diseases [9]. Wogonoside, one of the main active constituents isolated from *S. baicalensis,* has been proven to possess numerous pharmacological activities by *in vivo* and *in vitro* studies. This study was 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. (Shanghai, China); Dulbecco's modified eagle's medium (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/lewkmia-2 (Bcl-2) and Bcl-2 associated X protein (Bax), phosphorylated (p)-glycogen synthase kinase (GSK)-3β, 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).

Animals and ethics statement

Experimental BALB/C nude mice (5-6 weeks old) were obtained from the Southwestern Medical University Experimental Animal Center (Luzhou, China). All the animal protocols were strictly performed with Laboratory Animal Care and Use Guidelines of National institute of Health [10], and were approved by the ethics committee of Animal Care and Use Committee of the Southwest Medical University (No. A 20190022-013).

Cell culture

Human bladder cancer 5637 cell line was acquired from American Type Culture Collection (Manassas, VA, USA), which were cultured in humidified atmosphere (37 °C, 5 % CO_2) 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 [11]. 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 μM for another 24 h.

The microplate reader (Bio-rad, CA, USA) was employed to measure at 570 nm, and the IC_{50} value of wogonoside against 5637 cells was calculated. Furthermore, to investigate the anti-proliferative effect of wogonoside in time-dependent manner, 5637 cells were treated with wogonoside at 8, 16, and 32 μM 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.

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

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

The anti-tumor effect of wogonoside *in vivo*

In this study, 12 nude mice were divided into control and wogonoside (30 mg/kg, i.p.) group (n = 6). Tumor-bearing mice were prepared according to the previous reports 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 injected 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 nude mice were determined using a Vernier caliper within 15 days (every 5 days on average), and the tumor volumes were calculated as follows: Tumor volume = $(\text{width}^2 \times \text{length})/2$ [12].

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, which were then exposed to wogonoside at concentrations of 8, 16, and 32 μM. Subsequently, the bladder cancer 5637 cells were collected for ELISA experiments to determine caspase-3, caspase-9 levels as the manufacturers' instructions.

Western blotting assay

The human bladder cancer 5637 cell lines or tumor tissues were collected, and the proteins were extracted using RIPA lysis buffer. After determination of protein concentrations using a BCA kit, equal amount of 30 μg proteins were parted with SDS-PAGE. The primary antibodies of Bcl-2 (1:1000), Bax (1:2000), p-GSK-3β (1:1000), p-ERK1/2 (1:1000), and p-AKT (1:1000) were incubated overnight at 4°C, which were then incubated with the HRP-conjugated secondary antibodies for 2 h at room temperature. The specific bands were analyzed using an ECL chemiluminescence kit. GAPDH was used as the internal reference for normalizing protein.

Statistical analysis

All the data are presented as the mean ± 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 the Figure 1 A, wogonoside showed a significant anti-proliferative activity, in a dose-dependent manner at 2 - 128 μM (Figure 1 A), against the human cancer 5637 cell line with an IC_{50} value of 20.59 μM. 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 effect of wogonoside on bladder cancer 5637 cells *in vitro*

As observed in the Figure 2, wogonoside at concentration of 8, 16, and 32 μM gradually increased 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 blotting assay (Figure 3). Wogonoside (8, 16, and 32 μM) obviously decreased the Bcl-2 level ($p < 0.01$) but increased the Bax ($p < 0.01$) in a concentration-dependent manner. These findings revealed that the anti-proliferative effect of wogonoside on bladder cancer 5637 cells might be associated with mitochondria-induced apoptosis.

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

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

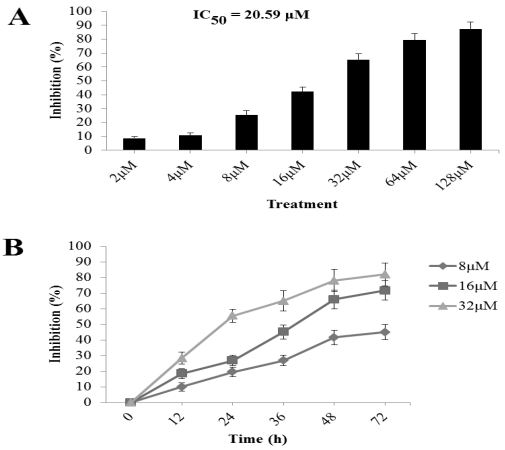

Figure 1: The anti-proliferative activity of wogonoside against the bladder cancer 5637 cell line. (A) The bladder cancer 5637 cells were exposed to wogonoside at 2 - 128 μM for 24 h. (B) The bladder cancer 5637 cells were treated with wogonoside at 8, 16, and 32 μM for 0, 12, 24, 36, 48, and 72 h. (n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

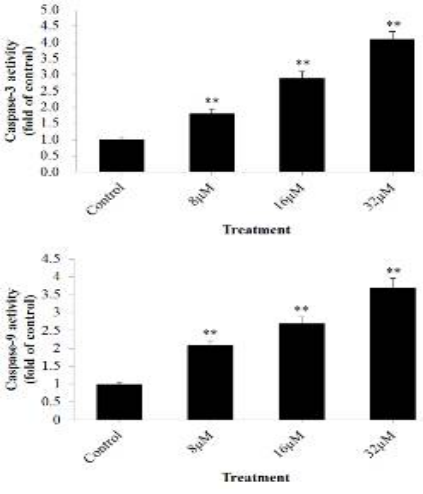

Figure 2: Effect of wogonoside (8, 16, and 32 μM) on caspase-3 and caspase-9 expression in 5637 cells. Data are represented as the mean ± SD (n = 4); $^{**}p < 0.01$ *vs.* the control

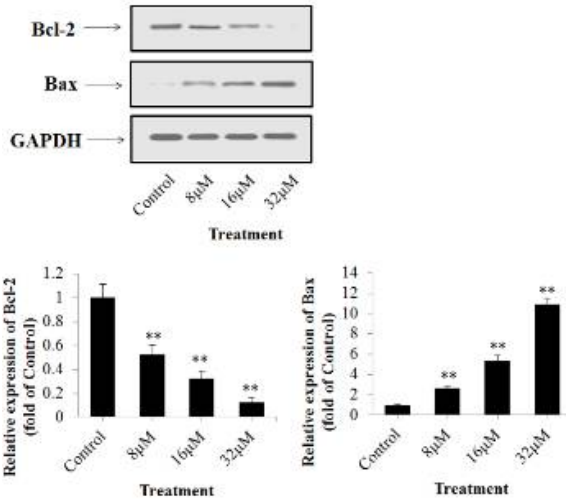

Figure 3: Effect of wogonoside (8, 16, and 32 μM) on Bcl-2 and Bax expression in 5637 cells. Data are represented as the mean ± SD (n = 4); ***p* < 0.01 *vs.* the control

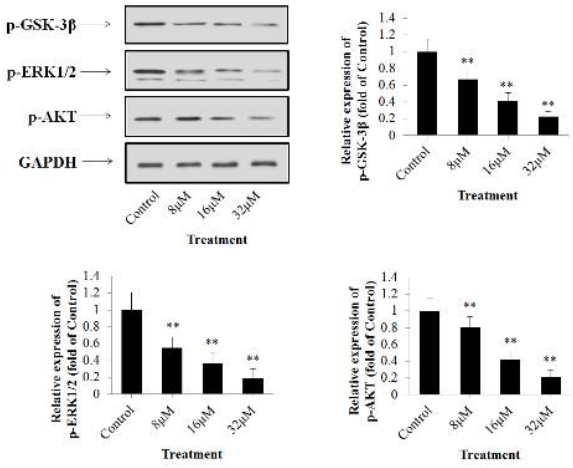

Figure 4: Effect of wogonoside (8, 16, and 32 μM) on p-GSK-3β, p-ERK1/2, and p-AKT expression in 5637 cells. Data are represented as the mean ± SD (n = 4); ***p* < 0.01 *vs.* the control

The anti-tumor effect of wogonoside on tumor-bearing mice

The *in vitro* results indicated that wogonoside exerts anti-proliferative effect against bladder cancer 5637 cell lines maybe *via* induction of the mitochondrial pathway of apoptosis. Furthermore, the anti-tumor effect of wogonoside on bladder cancer 5637 cells *in vivo* was investigated by replanting tumor on nude mice model. As shown in the Figure 5 A, wogonoside at dose of 30 mg/kg obviously suppressed tumor growth within a 15-day observation period ($p < 0.01$). However, 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 revealed that wogonoside exhibited an anti-tumor potential on mice bearing tumors of 5637 cells *in vivo*. As presented in the Figure 6, compared to the control mice, caspase-3 expression level was markedly increased in wogonoside-treated tumor-bearing mice ($p < 0.01$), whereas p-GSK-3β, p-ERK1/2, and p-AKT ($p < 0.01$) were decreased.

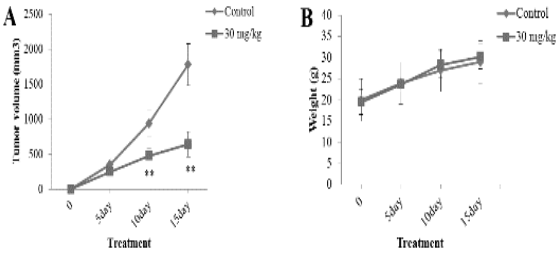

Figure 5: The anti-tumor activity of wogonoside (30 mg/kg) on nude mice bearing 5637 tumor cells. (A) Effects of wogonoside on tumor volume and (B) body weight of 5637 tumor-bearing mice. Data are represented as the mean ± SD (n = 6); ***p* < 0.01 *vs.* the control

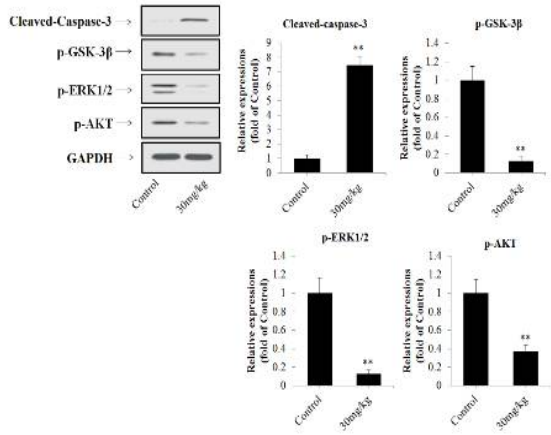

Figure 6: Effect of wogonoside (30 mg/kg) on expression of caspase-3, p-GSK-3β, p-ERK1/2, and p-AKT in tumor tissues. Data are represented as the mean ± SD (n = 6); ***p* < 0.01 *vs.* the control

DISCUSSION

Human bladder cancer is considered as the most serious cancer of the urinary system with an increasing morbidity and fatality [2]. However, long-term administration of the synthetic chemotherapeutics for treating bladder cancer, such as methotrexate, doxorubicin, and cisplatin, can lead to serious drug-resistances and toxicities [13]. The findings in this present study revealed that wogonoside possesses significant anti-tumor effect against human bladder cancer 5637 cells *in vivo* and *in vitro*, and the possible underlying mechanisms are related to regulation of the p-GSK-3β/p-ERK1/2/p-AKT signaling pathway.

Recently, it was reported that unregulated cell proliferation and apoptosis would result in the development of cancers [14]. Apoptosis or programmed cell death, a physiological cell suicide process, is recognized as an effective strategy for cancer treatment [15]. Cancer cell 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 [16-18]. The proportion between Bcl-2 and Bax protein 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 [19,20]. The western blotting assay indicated 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-tumor effect of wogonoside against human bladder cancer 5637 cells mainly through 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 cancer cell apoptosis and malignant transformation [21,22]. Thus, it is generally considered that blocking of the ERK1/2/MAPK signaling pathway could be beneficial for the treatmnet of bladder cancers in clinic. It is also reported that AKT protein is important for the PI3K/AKT signaling pathway, and inhibition of AKT phosphorylation is beneficial for treating cancers [23,24]. GSK-3β is another crucial protein in the AKT signaling pathway, and suppression of GSK-3β 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-3β 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 the GSK-3β/ERK/AKT signaling pathway. The present work provides a new insight into the pharmacological role of wogonoside that would be beneficial for the clinical treatment of bladder cancer.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

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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