The Anticancer Activities of Wogonin in Murine Sarcoma S180 both in vitro and in Vivo

Wei Wang, a Qing-Long Guo, a Qi-Dong You, b Kun Zhang, a Yong Yang, a Jun Yu, a Wei Liu, a Li Zhao, a Hong-Yan Gu, a Yang Hu, a Zi Tan, a and Xiao-Tang Wang a

a Department of Physiology, China Pharmaceutical University; b Department of Medicinal Chemistry, China Pharmaceutical University; No. 24 Tong Jia Xiang, Nanjing 210009, China; and c Department of Chemistry and Biochemistry, Florida International University; 11200 SW 8th St., Miami, FL 33199, U.S.A.

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The anticancer effects of wogonin on murine sarcoma S180 both in vitro and in vivo were investigated, and its pro-apoptotic molecular mechanism was further studied. Wogonin treatment resulted in significant inhibition of S180 cells in a concentration-dependent manner detected by MTT assay. The IC50 value for 48 h was (7.37 ± 1.53) × 10^-5 M. Typical morphological changes and apoptosis bleb phenomenon in S180 cells exposed to wogonin were distinctly observed by the inverted light microscope and the fluorescence microscope, respectively. According to protocols of transplanted tumor research, mice were transplanted with tumor cells S180. The weight of tumor and the peripheral leucocyte count were observed after the treatment of wogonin. The significant suppression of tumor growth was observed, and the peripheral leucocyte count of S180-bearing mice remained no significant changes compared with control group. After the treatment of 40 mg/kg wogonin, the inhibitory rate of tumor weight was 53.01%. Additional DNA fragmentation assay showed that wogonin induced apoptosis on murine sarcoma S180 tissue. RT-PCR results indicated that the increasing mRNA levels of bax and p53 and the decreasing mRNA level of bcl-2 were induced by wogonin. Western-blot assay showed that the increasing protein level of bax and the decreasing protein level of bcl-2 were induced by wogonin. Collectively, wogonin could induce apoptosis in murine sarcoma S180 thereby inhibiting the tumor growth both in vitro and in vivo. The pro-apoptotic effects might be related to the improvement of mRNA level of p53, the improvement of mRNA and protein levels of bax, and the reduction of mRNA and protein levels of bcl-2.

Key words wogonin; murine sarcoma S180; apoptosis; bcl-2; bax; p53

Apoptosis is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. 2) It plays a central role in controlling cell proliferation and hence is pivotal to the prevention of tumor development, 3) which is characterized morphologically by cytoplasmic shrinkage, plasma membrane blebbing, nuclear chromatin condensation, chromosomal DNA cleavage, and fragmentation of the cells into membrane-enclosed vesicles or apoptotic bodies. 4—6) In the recent years, the understanding of apoptosis process has provided the basis for novel, targeted therapies that specifically induce cell death in carcinoma cell lines or enhance the cytotoxic effects of established chemotherapeutic agents in these cells. 7,8) These novel agents include those targeting the extrinsic pathway such as tumor necrosis factor-related apoptosis-inducing ligand receptor 1, 9,10) and those targeting the intrinsic Bcl-2 family pathway such as antisense bcl-2 oligonucleotides. 11) Many pathways and proteins control the apoptosis machinery. Examples include p53, 12) the nuclear factor kappa B, 13) the phosphatidylinositol 3 kinase pathway, 14) and the ubiquitin/proteosome pathway. 15) These can be targeted by specific modulators such as bortezomib, 16) and mammalian target of rapamycin inhibitors such as CCI-779 and RAD 001. 17,18) Because these pathways may be preferentially altered in tumor cells, there is great potential for a selective effect in tumors sparing normal tissues provided an efficient and selective agent can be identified. 21

Wogonin (C16H12O5, Fig. 1), 19) one of the main active compounds of Scutellariae radix, is known to exert potent anti-inflammatory activities in vitro as well as in vivo. 20) Previous study revealed that it could inhibit lipopolysaccharide-induced production of nitric oxide (NO) and prostaglandin E2 in macrophages, inhibit cyclooxygenase-2 expression and alleviate skin inflammation in mice. 21—23) In addition, variable degrees of radical scavenging and antioxidant activities of wogonin were reported in many experimental systems. 24—26) Recently, some reports indicated that wogonin could induce apoptosis thereby significantly inhibiting human ovarian cancer cell A2780, human promyeloleukemic cell HL-60, and human hepatocellular carcinoma cells SK-HEP-1. 27—29) However, knowledge of the molecular mechanisms of wogonin-induced apoptosis was extremely scarce and remained to be delineated. This paper was undertaken to evaluate the inhibition and apoptosis induction of wogonin in murine sarcoma S180 both in vitro and in vivo, and the probable apoptotic molecular mechanisms. Results from our present study in vitro and in vivo indicated that wogonin had significant inhibition on S180 cells cultured in vitro and S180 transplanted tumor in ICR strain of mice. We further demonstrated, for the first time, the regulation of p53, bax and bcl-2 genes and proteins was involved in wogonin induced apoptosis of murine sarcoma S180.

MATERIALS AND METHODS

Medicine Wogonin was isolated from Scutellariae radix

Fig. 1. Molecular Structure of Wogonin (C16H12O5. MW: 284.27)
according to the protocols reported previously with slight modifications.30,31) Samples containing 99% or higher wogonin were used in all experiments unless otherwise indicated. In in vivo experiments, wogonin was dissolved in 0.9% normal saline and was kept at 4 °C; in in vitro experiments, the stock solution of wogonin (10−7 M) was prepared using 30 mM NaOH and was kept at −20 °C until needed, and final concentrations of NaOH were found to have no effect on cell growth.

Animal  ICR species mice, 18—22 g, half male and half female, were supplied by Experimental Animal Center, China Pharmaceutical University. Animals were kept under standard laboratory conditions: free access to standard laboratory food and tap water, constant room temperature of 22 °C, 50—60% humidity with a natural day–night cycle, etc. The animal care and surgery protocols were designed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health, U.S.A.

Tumor Cell Line  Murine sarcoma S180 cells provided by Jiangsu Institute of Antitumor Pharmaceuticals, were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin G, and 100 µ/ml streptomycin, pH 7.4 in an Water Jacketed CO2 incubator (Thermo Forma, U.S.A.) with a humidified atmosphere of 5% CO2 at 37 °C.

Reagents  MTT (Fluka, U.S.A.) was dissolved in 0.01 M PBS. AO (acridine orange), and EB (ethidium bromide) were acquired from Sigma. Cyclophosphamide (200 mg/amoule) was purchased from Hualian Pharmaceutical Company Ltd., Shanghai; Genomic DNA Purification Kit was purchased from Fermentas. PCR primers were synthesized by Sangon Shanghai (China). Primary antibodies were purchased from Santa Cruz Biotechnology Inc, and IRDyeTM 800 conjugated anti-goat and anti-rabbit second antibodies were obtained from Rockland Inc.

Cell Growth Inhibition Assessed by Colorimetric MTT-Assay  The logarithmic cells were dispersed with 0.02% (w/v) EDTA to prepare 2.5×105/ml cell suspension, and partitioned into wells of 96-well plates at 100 µl/well for 24 h culture in a 5% CO2 incubator under 37 °C. The cells were then exposed to wogonin of different concentrations (0.5, 1.0, 5.0, 8.0, 20, 60, 80, 100×10−5 M) (100 µl/well). After 48 h incubation, 5 mg/ml MTT solution (20 µl/well) was added and cultured for 4 h, then the supernatant was discarded and DMSO was added (100 µl/well) in, respectively. The suspension was placed on micro-vibrator for 5 min and the absorbance (A) was measured at 570 nm by the Universal Microplate Reader (EL800, BIO-TEK INSTRUMENTS INC.). Triplet experiments were performed in a parallel manner for each concentration point and the results were reported presented as mean±S.D. Cell inhibitory ratio was calculated by the following formula:

\[
\text{inhibitory ratio} \% = \left( \frac{A_{\text{control}} - A_{\text{treated}}}{A_{\text{control}}} \right) \times 100\%
\]

\(A_{\text{treated}}\) and \(A_{\text{control}}\) were the average absorbance of three parallel experiments from the treated and control groups, respectively. IC50 was taken as the concentration that caused 50% inhibition of cell proliferation and was calculated by SAS statistical software.

Cell Morphological Assessment  Exponential growth phase S180 cells treated with 8×10−5 M wogonin for 24 h or 48 h were detected under the inverted light microscope, then all the floating cells and the attached cells were harvested with 0.02% (w/v) EDTA. The cell suspension 95 µl was mixed with 5 µl of dye mixture containing AO (100 mg/l) and EB (100 mg/l) in PBS. The cells were observed immediately by the fluorescence microscope (OlympusIX51, Japan). The peak excitation wave length was 490 nm.32)

Effect of Wogonin on Growth of Transplanted Tumor S180 in Mice  ICR mice with body weight of 18—22 g were transplanted with S180 according to protocols of transplanted tumor research.1) After 24 h of tumor transplantation, model animals were at random divided into 5 groups, and each group contained 10 mice, half male and half female. The groups with wogonin treatment received three dosages (10, 20, 40 mg/kg), respectively. The positive group was treated with cyclophosphamide (20 mg/kg). The control group received 0.9% normal saline. All test drugs were given through injections 24 h after tumor transplantation (or inoculation). Treatments were done at a frequency of iv one time per day for a total of 7 consecutive days. Before each treatment, the mice of each group were weighed. After the treatments, all mice were killed and weighed simultaneously, and then tumor was segregated and weighed. Tumor inhibitory ratio was calculated by the following formula:

\[
\text{tumor inhibitory ratio} \% = \left( \frac{W_{\text{control}} - W_{\text{treated}}}{W_{\text{control}}} \right) \times 100\%
\]

\(W_{\text{treated}}\) and \(W_{\text{control}}\) were the average tumor weight of the treated and control mice, respectively.

Effect of Wogonin on the Peripheral Leucocyte Count in Mice with S180  As described above, mice with S180 were treated on at a frequency of i.v. one time per day for a total of 7 consecutive days. After the treatments, all mice of 5 groups were killed, and then the peripheral blood was collected from the eye socket vein. The total white blood cells were counted under the microscope.

DNA Fragment Assay of Murine Sarcoma S180 Tissue  Wogonin induced apoptosis of murine sarcoma S180 tissue was detected by DNA fragment assay. Fresh tumor tissue was collected and stored at −80 °C after the mice were treated with wogonin (20, 40 mg/kg) seven times intravenously. The positive drug was cyclophosphamide, with its dosage of 20 mg/kg. In this assay, DNA was extracted by Genomic DNA Purification Kit (Fermentas) according to the manufacturer’s instructions, and then the products were electrophoresed on a 1.5% agarose gel and observed by EB staining using Gel-Pro analyzer (Genegenius, Syngene).

Reverse Transcription-PCR Assay of Murine Sarcoma S180 Tissue  Fresh tumor tissue was collected after the mice were treated with wogonin (20, 40 mg/kg) seven times intravenously. The positive drug was cyclophosphamide, with its dosage of 20 mg/kg. Total RNA was extracted from the S180 tissue by TriPure Isolation Reagent (Roche, U.S.A.). The specific sets of primers and the target gene amplification conditions were shown in Table 1. All initial denaturations were at 94 °C for 5 min. Finally an additional extension step at 72 °C for 7 min was done. The PCR products were elec-
trophoresed on a 1.5% agarose gel and observed by EB staining using Gel-Pro analyzer (Genegenius, Syngene).

**Western-Blot Assay of Murine Sarcoma S180 Tissue**

Fresh tumor tissue was collected after the mice were treated with wogonin (20, 40 mg/kg) seven times intravenously. The positive drug was cyclophosphamide, with its dosage of 20 mg/kg. Proteins of tissue were isolated by lysis buffer (100 mM Tris–Cl, pH 6.8, 4% m/v) SDS, 20% (v/v) glycerol, 200 mM $\beta$-Mercaptoethanol, PMSF 1 mM, Aprotinin 1 mg/ml, Sigma) and measured using the Bradford assay with BioPhotometer (BioPhotometer 6131 GB/HK, Eppendorf) at 595 nm. Protein samples were separated onto 12% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto the PVDF membranes (Millipore). Immune complexes were detected with rabbit anti-Bax, rabbit anti-Bcl-2 and goat anti-actin as primary antibodies, IRDyeTM800 conjugated anti-rabbit and anti-goat second antibodies. The immunoreactive bands were detected with the Odyssey Scanning System (LI-COR inc., U.S.A.).

**Statistical Evaluation**

Data are shown as mean±S.D. Statistical analyses are performed using an unpaired, two-tailed Student’s t-test. All comparisons are made relative to untreated controls and significance of difference is indicated as *$p<0.05$ and **$p<0.01$.**

**RESULTS**

**Cell Growth Inhibition Assessed by Colorimetric MTT-Assay**

Concentration-dependent inhibition of wogonin on murine sarcoma S180 cells for 48 h was observed. IC$_{50}$ for 48 h was (7.37±1.53)$\times10^{-5}$ M. The results also indicated that when the concentrations of wogonin were lower than 0.5$\times10^{-5}$ M, the inhibition was weak (data not shown); when the concentrations ranged from 0.5$\times10^{-5}$ M to 100$\times10^{-5}$ M, the inhibitory effect became obvious in a concentration-dependent manner (see Fig. 2).

**Cell Morphological Assessment**

Under the inverted light microscope, the untreated S180 cells grew well and the skeletons were clear. Cells treated with 8$\times10^{-5}$ M wogonin for 24 h were equivocal and structure-elongated and some cells were even broken. Majority of cells treated for 48 h were broken and necrosed (see Fig. 3).

Under the fluorescence microscope, S180 cells treated with 8$\times10^{-5}$ M wogonin for 24 h presented the morphological features of early apoptotic cells, such as bright condensed chromatin identified by AO staining and typical apoptotic bleb phenomenon. After incubation for 48 h, the typical apoptotic bleb phenomenon appeared, and the late apoptotic cells were observed by EB staining (see Fig. 4).

**Effect of Wogonin on Growth of Transplanted Tumor S180 in Mice**

After the seven-day treatments by the route of i.v., wogonin (20, 40 mg/kg) and cyclophosphamide (20 mg/kg) had significant inhibitory effect on the growth of inoculated S180 in mice. The inhibitory rates were 34.24%, 53.01% and 62.65%, respectively. Meanwhile, there was no significant difference in the average weight of wogonintreated mice compared with control mice; nevertheless, cy-

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Table 1. Primer Sequences for PCR and Amplification Conditions for Each Target Gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Amplification conditions</th>
<th>Product (base)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>F: AGGATGCGTCCACCAA</td>
<td>Denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and synthesizing at 72 °C for 45 s for 30 cycles</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>R: TTTCAACGGGCTCGACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>F: TGGGATCTGGAGATGAAAC</td>
<td>Denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and synthesizing at 72 °C for 45 s for 36 cycles</td>
<td>368</td>
</tr>
<tr>
<td></td>
<td>R: GGAAGAAACTCAAGCCACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>F: CCAGGATTTGGAGATTTT</td>
<td>Denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and synthesizing at 72 °C for 45 s for 35 cycles</td>
<td>410</td>
</tr>
<tr>
<td></td>
<td>R: GACGAGGTACACCTCACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-Actin</td>
<td>F: GTCGTACCACAGGCATTGTGATGG</td>
<td>Changed according to different target genes</td>
<td>492</td>
</tr>
<tr>
<td></td>
<td>R: GGTGATACATGGGTCGTAACG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Clophosphamide (20 mg/kg) could significantly inhibit the weight of mice (see Table 2, Figs. 5, 6).

**Effects of Wogonin on The Peripheral Leucocyte Count in Mice with S180** As shown in Table 2, Fig. 7, compared with control, wogonin (10, 20, 40 mg/kg) had no significant influence on the peripheral leucocyte count in mice with S180, while very significant inhibition on the peripheral leucocyte count resulted from cyclophosphamide (20 mg/kg).

**DNA Fragment Assay of Murine Sarcoma S180 Tissue** DNA was isolated from the S180 tissues. The results of DNA integrity analysis by agarose electrophoresis showed that wogonin (40 mg/kg) and cyclophosphamide (20 mg/kg) caused the digestion of genomic DNA into ladders obviously (see Fig. 8).

**The mRNA Levels of Bcl-2, Bax and P53 in Murine Sarcoma S180 Tissue** As shown in Fig. 9 (A—D), after

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**Table 2. Effect of Wogonin on S180 in Mice by i.v. Route (Mean±S.D.) (n=10)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Weight of mice (g)</th>
<th>Weight of tumor (g)</th>
<th>WBC (10⁹/l)</th>
<th>Inhibitory rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-medication</td>
<td>Post-medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.10±0.65</td>
<td>28.60±1.26</td>
<td>1.00±0.11</td>
<td>19.28±5.46</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>20</td>
<td>18.50±0.72</td>
<td>21.60±0.97**</td>
<td>0.37±0.04**</td>
<td>10.68±2.00**</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>18.60±0.75</td>
<td>27.40±1.43</td>
<td>0.47±0.05**</td>
<td>16.04±3.44</td>
</tr>
<tr>
<td>Wogonin</td>
<td>20</td>
<td>18.30±0.61</td>
<td>27.70±1.06</td>
<td>0.66±0.07**</td>
<td>18.70±4.24</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>18.40±0.73</td>
<td>27.80±1.48</td>
<td>0.85±0.07</td>
<td>18.77±4.36</td>
</tr>
</tbody>
</table>

**Fig. 4. Wogonin Induced Apoptotic Morphological Changes of Murine Sarcoma S180 Cells Detected with a Fluorescence Microscope (×200)**

(A) Control (24 h); (B) after the S180 cells were treated with wogonin 8×10⁻⁵ M for 24 h, the early apoptotic cells could be observed: the cells were stained green with AO, their membrane were still in integrity, the nuclei exhibited bright condensed chromatin (arrow 1), and some cells displayed typical apoptotic bleb phenomenon (arrow 2); dead cells were stained aequalis red with EB (arrow 3); (C) control (48 h); (D) after treated with wogonin 8×10⁻⁵ M for 48 h, many cells displayed typical apoptotic bleb phenomenon (arrow 4); the late apoptotic cells could be observed: their nuclei exhibited condensed chromatin, and they were stained red with EB (arrow 5).

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mice with S180 were treated with wogonin (20, 40 mg/kg) and cyclophosphamide (20 mg/kg) seven times intravenously, wogonin could lower the level of bcl-2 mRNA and increase the levels of bax and p53 mRNA dramatically in a concentration-dependent manner. Meanwhile, cyclophosphamide could decrease the level of bcl-2 mRNA and had no significant influence on the levels of bax and p53 mRNA.

The Protein Levels of Bcl-2, Bax in Murine Sarcoma S180 Tissue As shown in Fig. 10, after mice with S180 were treated with wogonin (20, 40 mg/kg) and cyclophosphamide (20 mg/kg) seven times intravenously, wogonin could lower the level of bcl-2 protein and increase the level of bax protein dramatically in a concentration-dependent manner. Meanwhile, cyclophosphamide could decrease the level of bcl-2 protein and had no significant influence on the level of bax protein.

DISCUSSION

In our studies, wogonin isolated from Scutellariae radix had significant inhibition on murine sarcoma S180 both in vitro and in vivo. In vitro, S180 cells incubated with $8 \times 10^{-5} M$ wogonin for 24 or 48 h underwent apoptosis with typically apoptotic characteristics including ultrastructural changes of cytoplasm condensation, nucleus disruption and apoptotic bleb phenomena. In vivo, obvious DNA ladders were detected in murine sarcoma S180 tissue by DNA fragmentation assay; our results from PCR assay showed a decreasing mRNA level of bcl-2 and increasing mRNA levels of bax and p53 in murine sarcoma S180 tissue; western-blot assay showed that an increasing protein level of bax and a decreasing protein level of bcl-2 were induced by wogonin. All of these above suggested that wogonin could induce occurrence of apoptosis thereby inhibiting tumor growth. The pro-apoptotic effects might be related to the improvement of mRNA level of p53, the improvement of mRNA and protein levels of bax, and the reduction of mRNA and protein levels of bcl-2.

It is now well established that apoptosis is a complex biological process involving many pathways. Some evidence suggests that one of the most important regulators of the apoptotic pathway is the Bcl-2 family of genes such bcl-2 and bax genes. The Bcl-2 family includes proapoptotic members such as bax, bak, bad, bcl-xs, bid, bik, and hrk, and antiapoptotic members such as bcl-xl, bcl-w, bfl-1, and mcl-1. Antiapoptotic gene bcl-2 acts as a repressor of apoptosis by blocking the release of cytochrome-c, whereas proapoptotic gene bax acts as a promoter. These effects are more dependent on the balance between bcl-2 and bax than on bcl-2 quantity alone. Sato and his coworkers (1994) proposed two possibilities: (i) Bcl-2 induced a pathway that actively maintained cell survival, with bax serving as a negative regulator of bcl-2; (ii) Bax directly or indirectly generated cell death signals, bcl-2 being the dominant inhibitor of bax. Thus, bcl-2 and bax might be two critical factors of apoptotic process. As shown in our study, lowering the level of bcl-2 mRNA and increasing the level of bax mRNA might be one of molecular mechanisms.

The effect of an anticancer drug was determined in part by how readily the tumor cells undergo apoptosis. In this paper, the apoptosis induction of wogonin was confirmed in murine sarcoma S180 and the results also showed that wogonin induced apoptosis was mediated by p53, bax and bcl-2. It was reported that the tumor suppressor gene, p53, functioned as a cellular emergency response system to induce cell...
growth arrest or apoptosis.\textsuperscript{43,44} Our result of up-regulation of p53 gene may be a critical factor of apoptosis induction of wogonin. Several pathways mediated p53-induced apoptosis, and one of these involved the bax gene that was the p53 target and the proapoptotic member of the Bcl-2 family of genes.\textsuperscript{45–47} Bax could promote the cytosolic release of cytochrome c, which in turn, activated caspase 3, one of the key executors of apoptosis, and then apoptosis occurred.\textsuperscript{48,49} In addition, bax could bind with bcl-2 and inhibit its function of apoptosis suppression. So the up-regulation of p53 and bax genes illustrated that the mechanism of wogonin induced apoptosis in murine sarcoma S180 involved activated p53 and bax genes.

In this paper, we demonstrated that wogonin induced apoptosis in murine sarcoma S180 via the concerted modulation of p53, bax and bcl-2 genes and proteins. This finding revealed an interesting correlation between gene regulation and wogonin induced apoptosis, and provided a molecular basis for the development of naturally occurring monoflavonoids as novel anticancer agents for better management of human cancers.

At present, the majority of anti-tumor medicines had serious side effects accompanying their therapeutic effects. Traditional Chinese medicine with high activity and low toxicity might provide novel drugs for tumor therapeutics. In our in vivo experiments, wogonin had strong anti-tumor activity even at relatively low doses, and it had no significant influence on the body weight and the peripheral leucocyte count of mice with S180. In conclusion, our study suggested that wogonin had good perspectives as a potent and selective anti-tumor drug.

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