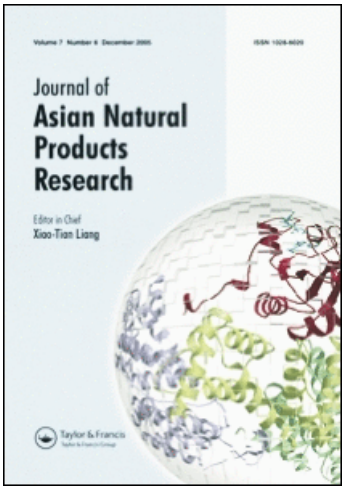

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[a] Anhui Key Laboratory of Modernized Chinese Material Medica, Anhui University of Traditional Chinese Medicine, Hefei, China [b] Department of Biomedical Sciences, Iowa State University, Ames, USA

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Apoptosis induced by baicalin involving up-regulation of P53 and bax in MCF-7 cells

Ning Wang[a], Li-Jian Tang[a], Guo-Qi Zhu[a], Dai-Yin Peng[a], Lei Wang[a], Fa-Neng Sun[b] and Qing-Lin Li[a]*

[a]*Anhui Key Laboratory of Modernized Chinese Material Medica, Anhui University of Traditional Chinese Medicine, Hefei, China;* [b]*Department of Biomedical Sciences, Iowa State University, Ames, USA*

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Anticancer effect of baicalin (**1**) has been well documented. However, the molecular mechanisms underlying the cytotoxicity of baicalin in cancer cells remain unclear. In the present study, we examined the potential roles of p53, bax, and bcl-2 in baicalin-triggered apoptosis in MCF-7 cells, a cell line derived from human breast cancer. The results showed that cell proliferation was significantly inhibited by baicalin in a dose- and time-dependent manner. Flow cytometric analysis also revealed that most of the baicalin-treated MCF-7 cells were arrested in the G_0/G_1 phase. Significant amount of cells underwent apoptotic cell death 24 h following baicalin treatment. Typical apoptotic characteristics such as chromatin condensation and the formation of apoptotic bodies were noted 48 h following baicalin exposure. Semi-quantitative analysis using RT-PCR revealed dramatic elevation of mRNA levels of proapoptotic molecules p53 and bax, but not the anti-apoptotic bcl-2. Consistently, significant elevation of p53 and bax was substantiated by the western blot. Collectively, the data demonstrated that baicalin-induced apoptotic cell death in the breast cancer cells involves the up-regulation of proapoptotic p53 and bax, implying potential crucial roles of bax and p53 in the baicalin-induced apoptosis.

Keywords: baicalin; MCF-7 cells; apoptosis; p53; bax

1. Introduction

Breast cancer is one of the devastating cancers endangering women's health, especially in the Western developed countries, with the incidence increasing over years. It was estimated that about 1.2 million more women are suffering from breast cancer worldwide yearly. Chemotherapy represents an important way in the clinical therapy of breast cancer. However, severe cytotoxicity of most of the commonly used anticancer drugs to normal cells necessitates the screening and development of some therapeutic reagents with relatively low side effect. In this regard, Chinese traditional herbs have been of particular interest, due to their relatively low toxicity as concluded from their extensive clinical usage in the past. Isolation and screening of the active components from the herbs possessing anticancer potential appears to be a promising way of discovering novel therapeutic compounds.

The epidemiological studies indicated that the incidence of breast cancer in Asian countries was much lower than that in the Western developed countries. Higher daily consumption of legumes has been suggested to be responsible, in part, for the low incidence of breast cancer in Asia, as Asia

*Corresponding author. Email: qinglin_lee@hotmail.com

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consumes 20 times higher amounts of the legumes than those of the Western developed countries, on average. In support, previous studies suggested that high abundant phytoestrogens present in soy could be the candidates exerting strong inhibitory effect on breast cancer [1]. Phytoestrogen, heterocyclic hydroxybenzene derived from plants, shares some structural similarities with estrogen, and thus could function as partial agonists for estrogen receptors. The phytoestrogen structurally falls into three groups: isoflavones, coumarins, or lignans. Numerous studies have revealed that phytoestrogen abstracted from the Chinese herbs could be an important source to acquire bioactive compounds for preventing the development of breast cancer. Diverse phytoestrogen-like substance including baicalin (**1**, Figure 1) has been isolated from the Chinese herb *Scutellaria baicalensis* Georgi. Experimental studies indicated that **1** possibly is the key bioactive ingredient mediating the anticancer effect of *S. baicalensis* Georgi. Compound **1** has been demonstrated to strongly inhibit the proliferation of bladder [2], prostate [3], liver [4], colon [5], and bile duct cancer cells [6] *in vitro*. This study intends to determine the cell death mechanisms by examining the potential roles of proapoptotic molecules, p53, bax, etc., in the baicalin-treated human breast cancer MCF-7 cells.

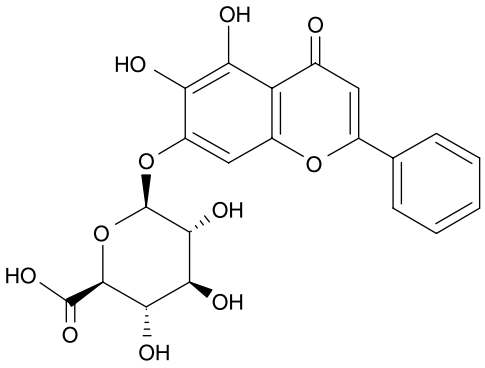


Figure 1. Structure of baicalin (**1**).

2. Results and discussion

First, we determine the effect of **1** on the proliferation of MCF-7 cells, a human breast cancer cell line, using the MTT assay. The results revealed strong inhibitory effect of **1** on cell proliferation (Figure 2). Exposure to **1** (50–400 μM) for 24 h results in the dose-dependent inhibition of cell proliferation, with IC_{50} at about 206 μM. Prolonged exposure (48 or 72 h) to indicated concentrations of **1** led to a similar dose-dependent reduction in the number of surviving cells, but to a much higher extent, with IC_{10} at 26 μM in 72 treatment paradigms. Apparently, at a concentration as high as 200 μM, **1** provoked strong cytotoxicity on MCF-7 cells, as high proportion of cells showed morphological alterations characteristic of cell death (data not shown). However, at low dose range (3–50 μM), **1** failed to elicit a significant cytotoxic effect on MCF-7 cells.

To explore whether apoptotic cell death was concerned with the cytotoxicity of **1**

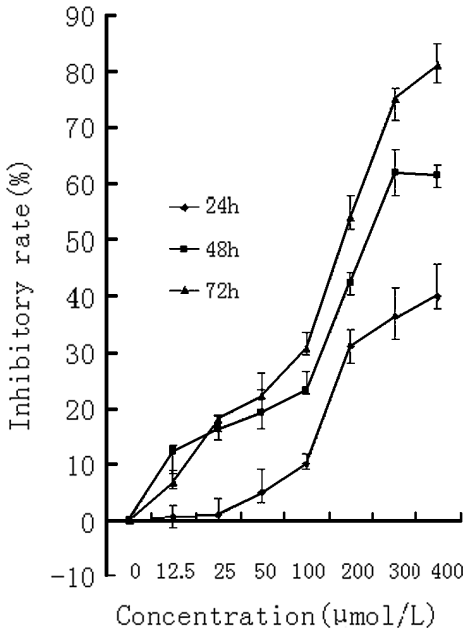


Figure 2. Time- and concentration-related effect of baicalin (**1**) on the inhibitory rate in MCF-7 cells. The inhibitory rate was assayed with the MTT assay. Data represent the means ± S.E. of six experiments.

Table 1. Effects of baicalin (**1**) on cell cycle distribution and apoptosis of MCF-7 cells[a].

	Percentage of cell cycle (%)			
	G0/G1	S	G2/M	
0 (Control)	59.80 ± 9.30	22.10 ± 3.30	15.10 ± 3.56	3.00 ± 0.46
50	65.05 ± 13.00	20.67 ± 3.56	8.36 ± 3.12	5.92 ± 1.12
200	67.10 ± 10.45	10.12 ± 2.21	8.88 ± 2.49	13.90 ± 2.35
400	62.60 ± 12.60	10.21 ± 2.09	6.49 ± 1.41	20.70 ± 5.34
5-Fu	45.33 ± 9.20	22.00 ± 3.12	6.95 ± 1.83	25.72 ± 4.96

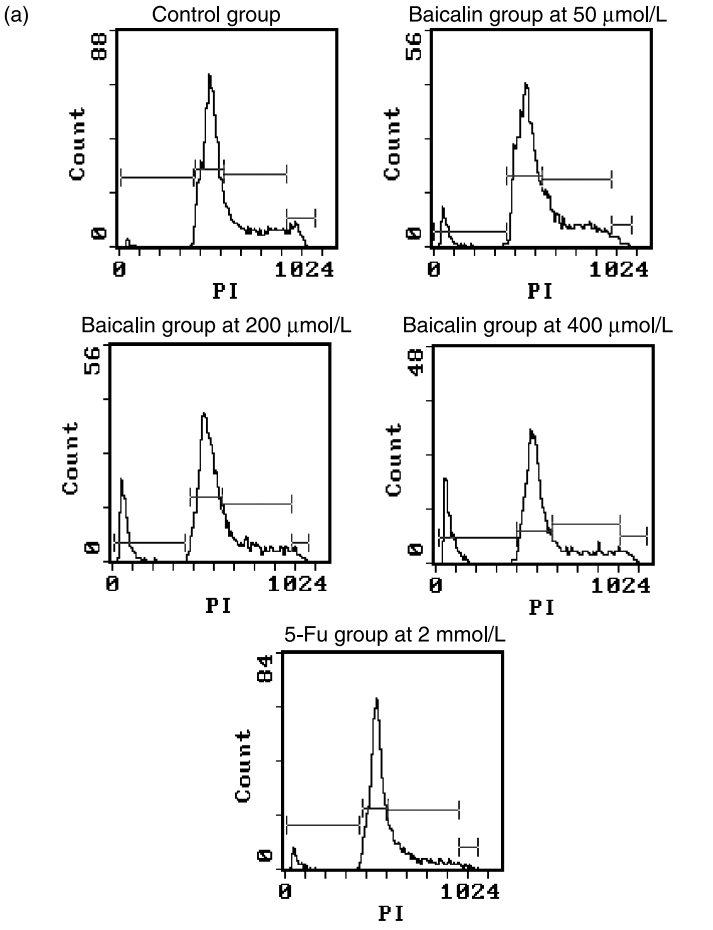
[a]All the data represent the means ± S.E. of triplicate experiments.

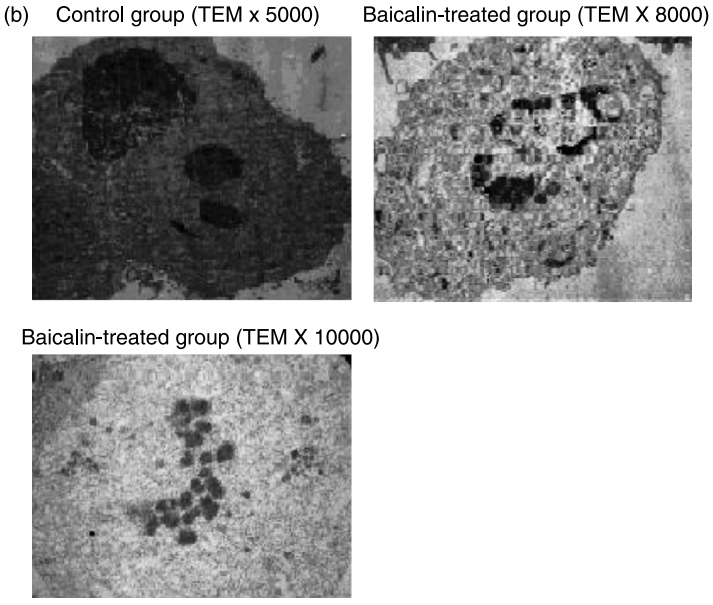
on MCF-7 cells, we conducted flow cytometric analysis to quantify the rate of cells undergoing apoptosis. As shown in Table 1 and Figure 2(A), 24-h post-exposure to **1**, 5.92, 13.90, and 20.70% of cells manifested apoptotic features at the 50, 200, or 400 μM groups, respectively, whereas only a minimal number of cells without drug treating (Table 1; Figure 2, control group, 3%) underwent apoptosis. Notably, the majority of **1**-treated cells were arrested in the G_0/G_1 phase. Collectively, the data suggested that **1** treatment resulted in the apoptotic cell death in the MCF-7 cells. In addition, extensive observation of chromatin condensation visualized under the electron microscope in **1**-treated cells further confirmed that apoptotic cell death represented the primary way of cell death triggered by **1** exposure (Figure 3(B)), as chromatin alteration is one of the characteristic features of the cells undergoing apoptotic cell death.

To examine the potential role of some proapoptotic molecules during baicalin-induced apoptosis, we performed RT-PCR to assay p53, bax, and bcl-2 expression. The results indicated that p53 expression was obviously up-regulated after **1** treatment (50, 200, 400 μM) for 48 h compared with the control groups ($P < 0.01$; Figure 4(A)), whereas no significant changes in bcl-2 mRNA level (Figure 4(C)). The similar significant up-regulation of bax ($P < 0.05$) was also noted with the same **1** treatment paradigm (50 and 200 μmol/L; Figure 4(D)). Surprisingly, high dose of **1** (400 μmol/L) failed to alter the expression of bax ($P > 0.05$; data not shown). Meanwhile, western blotting showed the same result in up-regulating p53 in the protein level as that done by RT-PCR (Figure 4(B)).

Scutellaria baicalensis Georgi (Huang Qin) is a widely used Chinese herbal medicine that has been used traditionally for anti-inflammatory and anticancer therapy. Baicalin (7-glucuronic acid, 5,6-dihydroxyflavone), a flavonoid compound isolated from *S. baicalensis* Georgi is one of its active components. Many studies had shown that it possesses antiproliferative effects on cancer cell lines of bladder [2], prostate [3], human hepatoma [4], colon [5], malignant melanoma [6] *in vitro*. However, little is known regarding its effect on breast cancer. Previous studies showed that alcohol extracts of *Radix scutellariae* had effect on the anti-growth of breast cancer MCF-7 cells *in vitro* [7]. In this study, first, we examined the effect of **1** on proliferation, cell cycle progression, and apoptosis. The MTT assay indicated that **1** significantly inhibited the proliferation of MCF-7 cells lines in a time- and dose-dependent manner. Analysis of cell cycle distribution and apoptosis using FCM showed that most MCF-7 cells treated with **1** were arrested in the G_0/G_1 phase, which may underlie the apoptosis of the MCF-7 cells.

Following **1** treatment, the cells were subjected to morphological changes, and the typically apoptotic characteristics such as condensed chromatin and apoptotic bodies were commonly observed under the electron microscope, further substantiating the undergoing apoptosis. To explore the apoptotic mechanism of breast cancer cells, we examined the related genes: p53, bcl-2, and





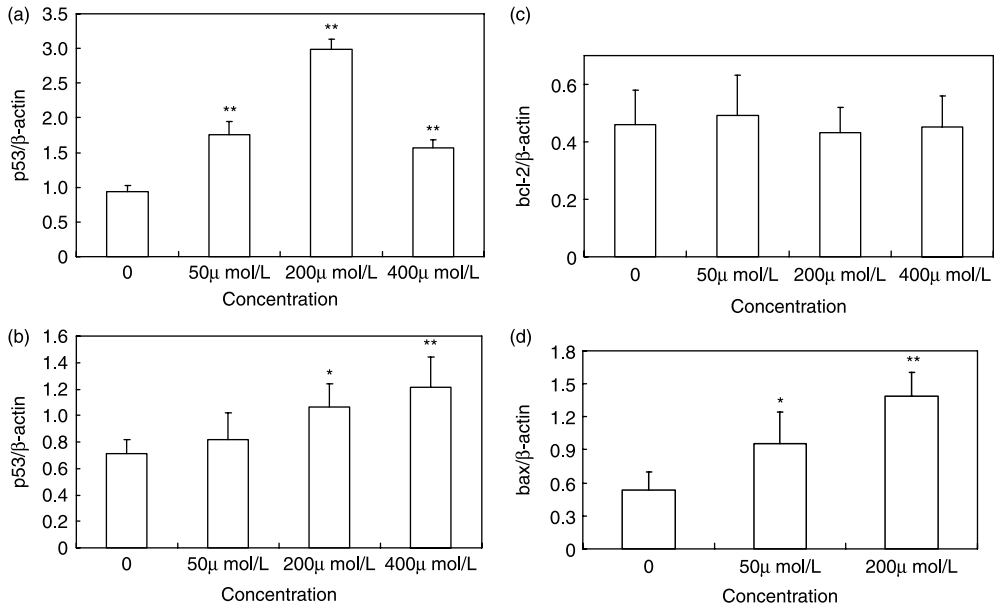


Figure 4. The effect of baicalin on the expression of WT p53, bcl-2, and bax gene in MCF-7 cells for 48 h. (a) The expression of WT p53 in mRNA level was determined by RT-PCR and (b) the result of which was in line with the expression in protein level determined by western blotting. (c) The mRNA expression of bcl-2 and (d) bax was also analyzed by RT-PCR method. All the data represent the means ± S.E. of triplicate experiments. Compared with the control group, $*P < 0.05$, $**P < 0.01$.

bax dependent on the methods of RT-PCR and western blotting. It was reported that the tumor suppressor gene, p53, functions as a cellular emergency response system to induce cell growth arrest or apoptosis [8,9]. Our result of increase in p53 protein may be a critical factor of apoptosis induction of **1**. Several pathways mediated p53-induced apoptosis, and one of these involved the bax proteins, which were the p53 targets and the preapoptotic member of the bcl-2 family of proteins [10–12]. Bax could promote the cytosolic release of cytochrome *c*, which in turn activates caspase 3, one of the key executioners of apoptosis, and then apoptosis occurred [13–15]. In addition, bax could bind with bcl-2 and inhibit its function of apoptosis suppression. In our study, there were little changes in the expression of bcl-2 mRNA, and the expression of WT-p53 mRNA, bax mRNA, and WT-p53 protein was increased. Consistently, the ratio of bax mRNA and bcl-2 mRNA was increased. **1** produced a notable protective effect on MCF-7 cells by up-regulating the expression of WT-p53 protein and WT-p53 mRNA, up-regulating bax mRNA expression, and regulating the balance of bax mRNA and bcl-2 mRNA to induce apoptosis. Therefore, the apoptosis induced by **1** was confirmed in MCF-7 cells and the mechanism of apoptosis induced by **1** was mediated by p53 and bax.

Figure 3. (a) Flow cytometry analyzed the cell cycle distribution and apoptotic rate of MCF-7 cells treated with baicalin (**1**) for 24 h. The variance between the baicalin-treated group and the control group was apparent. 5-FU was used as the masculine drug. (b) Morphological characteristics of MCF-7 cells were observed under the electron microscope. Treated with 200 μM baicalin, the cells had distinctly different characters compared with the control group. All the data represent the means ± S.E. of triplicate experiments.

3. Materials and methods

3.1 Cell culture

MCF-7 cells (human breast cancer cells) obtained from ATCC were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM L-glutamine, 50 U penicillin, and 50 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. The cells were cultured in corning 96-well plates for the MTT assay. For other flow cytometries, transmission electron microscopies, RT-PCRs, or western blotting analyses, the cells were grown in 6-well plates. Typically, the cells were subcultured every 3 days.

3.2 Assay for cell proliferation

Approximately, 2×10^4 cells were seeded in each well onto a 96-well plate. Logarithmic growth phase cells were treated with indicated concentrations of **1** (Shangxi, Jiangxi, China) for 24, 48, or 72 h. MTT (5.0 mg/mL; Sigma, St Louis, MO, USA) was then added to the culture medium and incubated for another 4 h at 37°C. After the removal of the culture medium, 150 μL DMSO was loaded onto each well to dissolve the precipitation. The absorbance (*A*) was measured at 490 nm using an Automated Microplate Reader (BIO-TEK ELx800UV; BioTek Instrument Inc., Winooski, VT, USA). The inhibitory rate of cell growth was defined as the percentage of the difference between the control and treated groups over the control group.

3.3 Flow cytometric analysis of apoptosis

Approximately, 5×10^6 cultured cells were harvested and washed twice with cold phosphate-buffered saline (PBS) and then incubated with PBS containing 500 mg/L PI and 50 mg/L RNase A for 30 min at 37°C. After washing twice with PBS, cell death was quantified using a flow cytometer (FACScan, Becton Dickinson, Canaan, CT, USA). Data were analyzed with CellQuest™ software (Becton Dickinson, San Jose, CA, USA).

3.4 Cell morphological assessment

Following exposure to **1** (200 or 400 μM) for 48 h, the cells were first fixed with 2.5% glutaraldehyde and post-fixed with 2% osmium tetroxide. After embedding the cells in EPOR812 (Nacalai Tesque, Kyoto, Japan), ultrathin sections (5 μm) were stained with lead citrate and uranyl acetate, and examined with a Hitachi-600 electron microscope (Hitachi, Tokyo, Japan).

3.5 Reverse transcriptase PCR

Total RNA was extracted using TRIzol Isolation Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (5 μg) was used as a template for cDNA synthesis, using the First-Strand kit of Novagen (Madison, WI, USA) by following the manufacturer's instructions. The resulting cDNA product (10%) was used in each PCR reaction. The primers specific for p53, bax, bcl-2, or β-actin (Sangon, Shanghai, China) as listed below. The PCR products were resolved on agarose gel, and the densitometric analysis was conducted for the quantification of individual band using Gel imaging Analyzer (Bio-Rad, San Diego, CA, USA).The following primers are used: 5′-GTGGAAGGAAATTTGCGTGT-3′ (p53 forward), 5′-CCAGTGTGATGATGGTGAGG-3′ (p53 reverse); 5′-GGTGCCACCTGTGGTCCACCTG-3′ (Bcl-2 forward), 5′-CTTCACTTGTGGCCCAGATAGG-3′ (Bcl-2 reverse); 5′-CAGCTCTGAGCAGATCATGAAGACA-3′ (Bax forward), 5′-GCCCATCTTCTTCCAGATGGTGAGC-3′ (Bax reverse); 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ (β-actin forward), 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′ (β-actin reverse).

The procedures are as follows: WT p53: 94°C 5 min; 94°C 45 s, 60°C 45 s, 72°C 2 min, 40 cycles; extension 7 min, 4°C paused; bcl-2: 94°C 5 min; 94°C 45 s, 60°C 45 s, 72°C 2 min, 40 cycles; extension 7 min, 4°C paused; bax: 94°C 5 min; 94°C 45 s, 60°C 45 s, 72°C 2 min, 40 cycles; extension 7 min, 4°C paused; β-actin: 94°C 30 s, 52°C 30 s, 72°C 1 min, 35 cycles; extension 5 min, 4°C paused.

3.6 Western blotting analysis

The cells were lysed with buffer (50 mM Tris–HCl, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 0.1% Triton X-100 plus protease/phosphatase inhibitor cocktail), and the cell lysates were centrifuged at 14,000 rpm to collect the supernatant. The protein concentration was determined using the Bradford assay. The cell lysates (20 μg) were separated on SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA), as described previously [7]. After blocking with 1% BSA, the membranes were incubated with p53 (1:1000; Boster, China) and HRP-conjugated secondary antibodies (1:10,000; Multisciences Biotech, Hangzhou, China). Enhanced chemiluminescence reagents (Biological Industries, Beit Haemek, Israel) were used for the detection of proteins of interest.

3.7 Statistical analysis

All the values were presented as means ± S.E. Statistical comparisons were conducted using either one-way ANOVA for multiple comparisons or Student's *t*-test for single comparisons. Statistical significance is accepted only if $P < 0.05$.

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References

[1] L.C. Zhao, L.J. Tang, Q.L. Li, and J.G. Liu, *China J. Chin. Mater. Med.* **31**, 57 (2006).
[2] S. Ikemoto, K. Sugimura, N. Yoshida, R. Yasumoto, S. Wada, K. Yamamoto, and T. Kishimoto, *Urology* **55**, 951 (2000).
[3] R.K. Tiwari, J. Geliebter, V.P. Garikapaty, S.P. Yedavelli, S. Chen, and A. Mittelman, *Int. J. Oncol.* **14**, 713 (1999).
[4] Y. Motoo and N. Sawabu, *Cancer Lett.* **86**, 91 (1994).
[5] S. Kuntz, U. Wenzel, and H. Daniel, *Eur. J. Nutr.* **38**, 33 (1999).
[6] W. Liu, M. Kato, A.A. Akhand, A. Hayakawa, M. Takemura, S. Yoshida, H. Suzuki, and I. Nakashima, *Int. J. Oncol.* **12**, 1321 (1998).
[7] L.J. Tang, L.C. Zhao, J.J. SU, F. Xiao, H. Cheng, L. Wang, G.Q. Zhu, Q.L. Li, and J. Anhui, *Univ. Trad. Chin. Med.* **25**, 23 (2006).
[8] W.S. El-Deiry, T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein, *Cell* **75**, 817 (1993).
[9] J.Y. Hsia, C.Y. Chen, C.P. Hsu, S.E. Shai, S.S. Yang, C.Y. Chuang, P.Y. Wang, and J.T. Chen, *Neoplasma* **48**, 483 (2001).
[10] G.J. Zhang, I. Kimijima, M. Onda, M. Kanno, H. Sato, T. Watanabe, A. Tsuchiya, R. Abe, and S. Takenoshita, *Clin. Cancer Res.* **5**, 2971 (1999).
[11] J.R. Streffer, A. Rimner, J. Rieger, U. Naumann, H.P. Rodemann, and W. Michael, *Neuro-Oncol.* **56**, 43 (2002).
[12] N. Bandoh, T. Hayashi, K. Kishibe, M. Takahara, M. Imada, S. Nonaka, and Y. Harabuchi, *Cancer* **94**, 1968 (2002).
[13] S.W. Lowe and A.W. Lin, *Carcinogenesis* **21**, 485 (2000).
[14] C. Communal, M. Sumandea, P. de Tombe, J. Narula, R.J. Solaro, and R.J. Hajjar, *Proc. Natl Acad. Sci. USA* **99**, 6252 (2002).
[15] C.F. Gaoa, S. Rena, L. Zhangb, T. Nakajimaa, S. Ichinosec, T. Harad, K. Koiked, and N. Tsuchida, *J. Exp. Cell. Res.* **265**, 145 (2001).