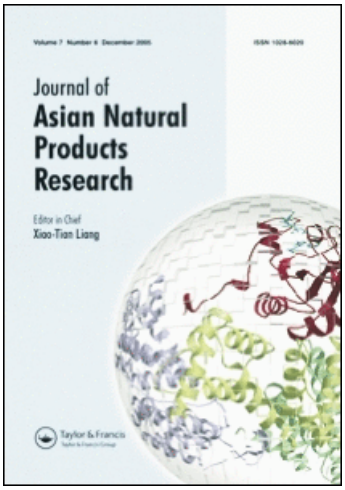


Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Effect of proliferation, cell cycle, and Bcl-2s of MCF-7 cells by resveratrol

Yi-Zheng Wang[ab]; Bo Cao[b]; Shao-Xiong Li[b]; Zhao-Hui Zang[b]; Jing-Ze Zhang[b]; Hong Chen[ab]
[a] Tianjin Key Laboratory for Biomarkers of Occupational and Environmental Hazard, Tianjin, China [b] Department of Pharmacognosy, Medical College of Chinese People's Armed Police Forces, Tianjin, China

To cite this Article Wang, Yi-Zheng , Cao, Bo , Li, Shao-Xiong , Zang, Zhao-Hui , Zhang, Jing-Ze and Chen, Hong(2009) 'Effect of proliferation, cell cycle, and Bcl-2s of MCF-7 cells by resveratrol', Journal of Asian Natural Products Research, 11: 4, 380 — 390
To link to this Article: DOI: 10.1080/10286020902772096
URL: http://dx.doi.org/10.1080/10286020902772096

Journal of Asian Natural Products Research
Vol. 11, No. 4, April 2009, 380–390

Taylor & Francis
Taylor & Francis Group

Effect of proliferation, cell cycle, and Bcl-2s of MCF-7 cells by resveratrol

Yi-Zheng Wang[ab], Bo Cao[b], Shao-Xiong Li[b], Zhao-Hui Zang[b], Jing-Ze Zhang[b] and Hong Chen[ab]*

[a]*Tianjin Key Laboratory for Biomarkers of Occupational and Environmental Hazard, Tianjin 300162, China;* [b]*Department of Pharmacognosy, Medical College of Chinese People's Armed Police Forces, Tianjin 300162, China*

(*Received 3 September 2008; final version received 18 January 2009*)

Our goals were to examine the dual-directional regulation effects of resveratrol (**1**) *in vitro* by using MCF-7 cells (estradiol receptor-positive cells), study its mechanism of action, and give a systematical analysis of the regulatory networks of each related factor. An MTT test and growth curve showed that the proliferation of MCF-7 cells was inhibited by a high concentration of **1**, and that its IC_{50} was $8.70 \times 10^{-5} \pm 0.23$ mol/l. However, **1** induced the proliferation of MCF-7 cells at 10^{-7}–10^{-5} mol/l, and resulted in a peak proliferation at 1.0×10^{-7} mol/l. A high concentration of **1** arrested cell cycle progression at the G_1 phase, and a typical "sub-G_1 peak" of apoptotic cells was also observed by flow cytometry. The proliferation index of MCF-7 cells increased significantly with a low concentration of **1** ($p < 0.05$). **1** in high concentrations induced Bax, caspase-3, and cyclin-dependent kinase (CDK) inhibitor P21 expression, whereas the expressions of cyclin CDK2, Bcl-2, and proliferating cell nuclear antigen (PCNA) were decreased by **1** treatment. Conversely, treatment with low concentrations of **1** decreased the expression of P21 and Bax, while the expressions of cyclin CDK2, Bcl-2, and PCNA were increased. These results suggest that **1** had a dual-regulatory effect on MCF-7 cells. CDK-associated protein was a key factor at both the high and low concentrations used in this study.

Keywords: resveratrol; MCF-7 cell; proliferation; apoptosis; cell cycle

1. Introduction

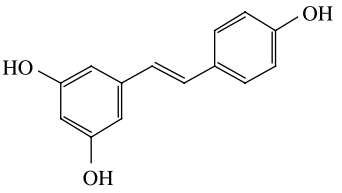
Phytoestrogens (PEs) as a non-steroid plant source have estrogenic activities. They combine with steroidal estrogen receptors (ERs) with low affinity to play a weak estrogen-like role [1]. PEs are estrogen agonists and antagonists, which play a role in the prevention and treatment of the diseases of postmenopausal women caused by estrogen reduction and other hormone-related conditions [2,3].

PEs include isoflavones, lignans, coumarins, mycoestrogens, and stibenes. Resveratrol (**1**), *trans*-3,5,4′-trihydroxystilbene (Figure 1), is a kind of natural polyphenolic compounds whose structure is similar to diethylstilbestrol. It exists widely in grapes, peanuts, and giant knotweed rhizomes. Lots of epidemiological studies and experimental results indicated that **1** has extensive medical treatment and healthcare functions, i.e., the effect of anti-platelet aggregation, low density lipoprotein-oxidation inhibitory, reducing blood lipid, free radical scavenging activity, diminishing inflammation, improving microcirculation, hepatic-protective, anti-cancer, and anti-inflammatory properties [4]. Recently, people pay more and more close attention to the phytoestrogenic-like activity of **1**. Here, we examine the phytoestrogen-like

*Corresponding author. Email: chenhongtian06@yahoo.com.cn

ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020902772096
http://www.informaworld.com



1

Figure 1. The structure of **1**.

effect of **1** using *in vitro* tests to study the mechanism. Recently, people pay more and more attention to **1**, and there are many reports about its anti-tumor effects and antioxidation effects. This paper selected MCF-7 cells (ER-positive cells) to examine the dual-directional regulation effects of **1** *in vitro* to study its mechanism of action, and gave a systematical analysis of the regulatory networks of each related factor. We hope our work could have guiding significance for further study.

2. Results and discussion

2.1 Effect of 1 on the proliferation of MCF-7 human breast cancer cells

The MTT assay showed that low concentration of **1** was beneficial to the proliferation of MCF-7 cells, and it resulted in a peak proliferation at 1.0×10^{-7} mol/l. However, the proliferation of MCF-7 cells was inhibited by high concentrations, and the IC_{50} was $8.70 \times 10^{-5} \pm 0.23$ mol/l. Furthermore, the results of the cell growth curve of MCF-7 cells matched with the above results. The effects were concentration and time dependent for the **1** treatment (Figure 2; Table 1).

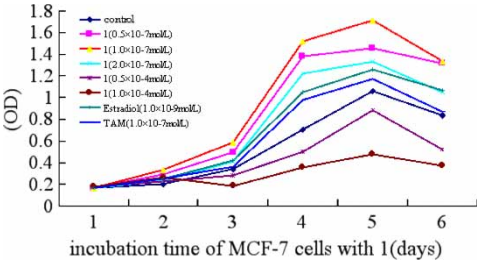


Figure 2. Time-dependent effects of **1** on MCF-7 cell growth and viability ($\bar{\chi} \pm s$, $n = 3$).

2.2 Effect of 1 on cell cycle progression

Cells were treated with the indicated concentrations of **1** (0.5, 1.0×10^{-4} mol/l; 0.5, 1.0, 2.0×10^{-7} mol/l) for 24 and 48 h, respectively, and distinct changes in cell cycle distribution were observed. At the lower concentrations ($0.5–2.0 \times 10^{-7}$ mol/l) of **1**, flow cytometric analysis showed that the DNA contents of S phase were more than that for the control, and that the value of proliferation index (PI) reached a maximum at 1.0×10^{-7} mol/l **1** in MCF-7 cells (47.0% 24 h and 56.9% 48 h) ($p < 0.05$) (Table 2). However, at higher concentrations ($0.5–1.0 \times 10^{-4}$ mol/l), the cells accumulated significantly in the G_1 phase of the cell cycle at 24 h (61.3% for control; 62.1% for 0.5×10^{-4} mol/l **1**; 77.3% for 1.0×10^{-4} mol/l **1**). At 48 h, cell cycle arrest in G_1 phase was more pronounced (57.5% for control; 74.8% for 0.5×10^{-4} mol/l **1**; 82.0% for 1.0×10^{-4} mol/l **1**). The sub-G_1 peak, an indicator of apoptotic cell death, was observed in the cells tested with higher concentrations. These results suggest that the anti-proliferative effect of **1** was related to the induction of cell apoptosis and the induction of cell cycle arrest at the G_1 phase by the high concentration of **1**, and occurred in a concentration- and time-dependent manner (Figure 3; Table 1).

2.3 Effect of 1 on the expression of proliferating cell nuclear antigen (PCNA), Bcl-2, and Bax mRNA of MCF-7 cells

Cells were treated with various concentrations of **1** (1.0×10^{-4} mol/l; 0.5, 1.0, 2.0×10^{-7} mol/l) for 48 h. The PCNA mRNA levels upon **1** treatment (0.5, 1.0, 2.0×10^{-7} mol/l) were $(78.02 \pm 2.15)\%$, $(88.37 \pm 8.93)\%$, and $(82.44 \pm 4.59)\%$, and was $(29.05 \pm 11.35)\%$ when treated by **1** (1.0×10^{-4} mol/l), while the corresponding mRNA level was $(69.09 \pm 0.74)\%$ in the

Table 1. The effect of **1** at high concentration on the cell cycle of MCF-7 ($n = 3$, %, $\bar{\chi} \pm s$).

Concentration (10^{-4} mol/l)	Ratio of cell population in different phases (%)						Ratio of apoptotic cells (%)	
	G_1		S		$G_2 + M$			
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	61.3 ± 3.17	57.5 ± 1.04	24.8 ± 4.98	33.4 ± 7.45	13.9 ± 7.23	9.1 ± 6.50	4.94 ± 0.96	7.52 ± 1.92
1 (0.5)	62.1 ± 6.74	74.8 ± 8.35*	31.6 ± 0.98*	22.0 ± 6.16*	6.3 ± 5.67	3.2 ± 3.05	17.7 ± 1.04*	28.5 ± 3.05*
1 (1.0)	77.3 ± 1.49*	82.0 ± 1.21*	22.6 ± 1.40	10.3 ± 1.93*	0.1 ± 0.10*	7.7 ± 0.72	31.1 ± 2.07*	38.6 ± 4.65*

Compared with control group.
* $p < 0.05$.

control group (Figure 4). There were significant differences in PCNA mRNA expression between the control group and both experimental concentration groups (1.0×10^{-4} and 1.0×10^{-7} mol/l, $p < 0.05$). At high concentrations, **1** down-regulated the expression of PCNA, inducing the apoptosis of MCF-7 cells. Conversely, at low concentrations, **1** up-regulated the expression of PCNA, promoting the proliferation of MCF-7 cells. **1** treatment had the same effect on Bcl-2 mRNA expression as PCNA, while the effect on Bax mRNA expression was the opposite (Figures 5 and 6). The expression levels of the three genes were all concentration dependent (Figure 7).

2.4 Effect of 1 on the expression of regulating proteins

Cells were treated with various concentrations (0.5, 1.0×10^{-4} mol/l) of **1** for 48 h. To examine the effects of high concentrations of **1** on the expression of proteins related to the regulation of cell cycle progression, total cell lysates were analyzed by western blot. As shown in Figure 7, P21, the CDK inhibitor, was induced by a treatment with **1** for 48 h. Down-regulation of CDK2, which promotes cell cycle progression from the G_1 phase into the S phase, was also observed in a concentration-dependent manner at 24 h (Figure 9). In addition, a concentration-dependent increase of caspase-3, which is downstream of cell apoptosis and promotes apoptosis progression, was observed by treatment with **1** for 48 h (Figure 8).

Based on our experimental results and relative literature data, we could draw an inference as follows: when exogenous estrogen absent, low concentrations of **1** combining with ERα in MCF-7 cells starts up the classical ER-mediated function way [5] (after combining with the ligand, ER changes its configuration, separates from the molecular chaperone heat-shock protein, and forms the homodimers or heterodimers. Then the dimers combine with the ER-responsive

Table 2. The effect of **1** at low concentration for 24 h on the cell cycle of MCF-7 ($n = 3$, %, $\bar{\chi} \pm s$).

Concentration (10^{-7} mol/l)	Ratio of cell population in different phases (%)						PI (%)	
	G_1		S		$G_2 + M$			
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	61.3 ± 2.48	57.5 ± 2.59	24.8 ± 1.35	33.4 ± 4.11	13.9 ± 1.54	9.1 ± 1.56	38.7	42.4
1 (0.5)	53.9 ± 2.41*	45.9 ± 5.96*	30.6 ± 0.80*	42.2 ± 3.76*	15.5 ± 1.61	11.9 ± 5.66	46.1	54.1
1 (1.0)	53.0 ± 1.77*	43.1 ± 2.98*	28.3 ± 0.96*	43.7 ± 1.10*	18.7 ± 1.74*	13.2 ± 1.93	47.0	56.9
1 (2.0)	54.9 ± 1.93*	48.2 ± 1.15*	32.5 ± 0.96*	41.2 ± 1.11*	12.6 ± 2.72	10.6 ± 0.17*	45.1	51.8
Estradiol (0.01)	57.4 ± 1.32	52.4 ± 3.21	32.0 ± 2.12*	31.9 ± 2.63	10.6 ± 1.85	15.7 ± 0.78*	42.6	47.6
Tamoxifen (TAM) (1.0)	60.3 ± 2.90	57.7 ± 3.59	28.9 ± 2.86*	29.0 ± 1.31	10.8 ± 0.79	13.3 ± 2.34	39.7	42.3

Compared with control group.
* $p < 0.05$.

element of the target gene, and influence the gene transcription and translation). At the same time, the expression of PCNA and Bcl-2 was up-regulated by low concentrations of **1** in MCF-7 cells. PCNA as a cycle protein is associated with the cell proliferation, while *Bcl-2* gene can block apoptosis without influencing the cell proliferation. Therefore, the effect of promoting proliferation of MCF-7 cells by low concentrations of **1** was closely related to PCNA. Up-regulated PCNA gene promotes DNA replication, and accelerates the cells in G_0/G_1 phase entering into S phase [6].

When high concentrations of **1** act on MCF-7 cells, it may, on the one hand, compete with endogenous estrogen and bind ERs to weaken the response of target cell to estrogen; thus playing an anti-estrogen role. On the other hand, PEs have far higher affinity for ERβ than ERα [7], after ERβ combining with **1**, the AP1-response element of target genes inhibits gene transcription by influencing ER-mediated transactivation activity, in opposition to the ERα-mediated mode of action. At the same time, by high concentrations of **1** among the genes whose expression was down-regulated were PCNA and Bcl-2, and whose expression was up-regulated were P21 and Bax. Bax is a widely studied protein in the Bcl-2 family. Bax is located in the cytoplasm, and is the apoptosis-induced gene forming Bax/Bax homodimers. When the cells increase Bcl-2, Bcl-2 and Bax form more of a heterologous dimer, weakening the apoptotic trend; whereas, when the cells have more Bax, Bax itself forms a dominant homologous dimer, making the cell more prone to apoptosis. The ratio of Bcl-2/Bax determines whether the cells can enter a state of apoptosis or not. Caspase-3 is the key in the pathway of apoptosis [8,9], and acts as the effecter or executioner of apoptosis [10,11]. High concentrations of **1** up-regulate the expression of the caspase-3 protein; thereby inducing the apoptosis of MCF-7 cells.

It can, therefore, be assumed that there may be two ways for high concentrations of **1**

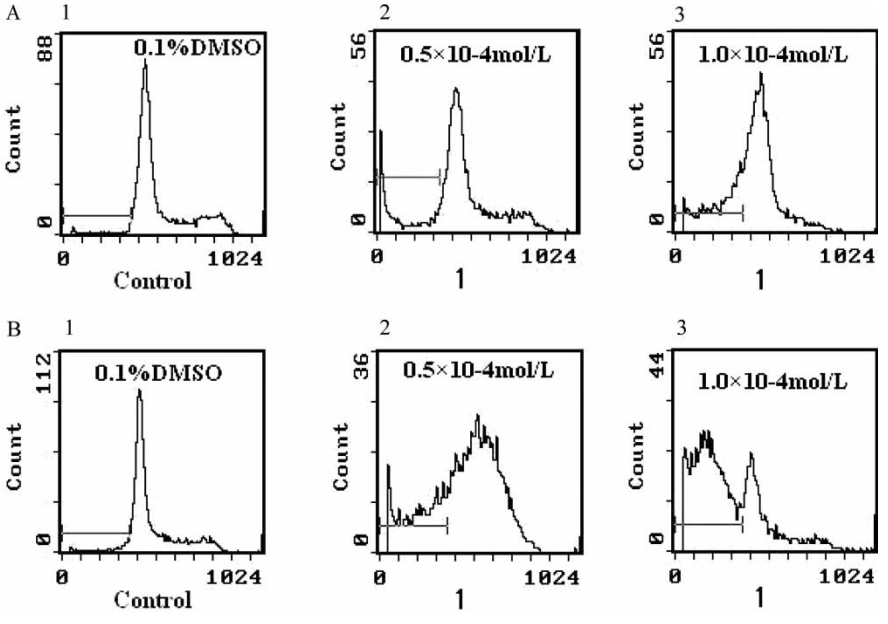


Figure 3. Demonstration of apoptosis by flow cytometric analysis. 1, untreated MCF-7 cells; 2 and 3, appearance of cells with sub-diploid DNA content after exposed to increasing concentrations of **1** (0.5×10^{-4} and 1.0×10^{-4} mol/l) for A, 24 h and B, 48 h.

to induce apoptosis in MCF-7 cells: first, by influencing the Bcl-2/Bax ratio, changing the cell's mitochondrial membrane-associated protein conformation, losing the cell function, releasing of various apoptosis-related proteins, and finally leading to apoptosis; second, by leading to cell cycle arrest. In normal cells, the P21 protein and the cyclin, CDK, and PCNA form a cyclin–CDK^4/$p21^4$/PCNA tetramer compound. When the expression was up-regulated, P21 protein combines with the CDK2–cyclin compound, inhibits the protein kinase activation, and arrests the cell cycle [12]. P21 simultaneously combines with PCNA, blocking DNA replication. The cell proliferation signal cannot be formed effectively and result in a cell block, thus the cells have sufficient time for damage repair.

It can be speculated that **1**'s role in MCF-7 cells proliferation/apoptosis is influenced by the Bcl-2 family of genes, cell cycle regulatory proteins, and the caspase-3 protein. High and low concentrations of **1** had opposite effects on MCF-7 cells.

Thus, we can see that **1** has a bimodal regulatory effect on MCF-7 cells, showing the characteristics of plant estrogen and having potential as a natural herbal extract (Figure 10).

3. Materials and methods

3.1 Chemicals

MTT and DMSO were purchased from Sigma Chemical Co. (St Louis, MO, USA). RPMI1640, non-phenolsulfonphthalein RPMI1640, and trypsin were the products of Gibco-BRL (Grand Island, NY, USA). CDT-FBS was purchased from Hyclone Co. (Logan, Utah, USA) Trizol was purchased from Invitrogen Co. (California, USA) The RT-PCR kit was purchased from Takara Shuzo Co., Tokyo, Japan. PCNA, Bcl-2, and Bax primers were the products of Shanghai Biotechnology Co., Shanghai, China. Mouse monoclonal anti-P21, anti-caspase-3 antibody, rabbit monoclonal anti-CDK2 antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, and HRP-conjugated

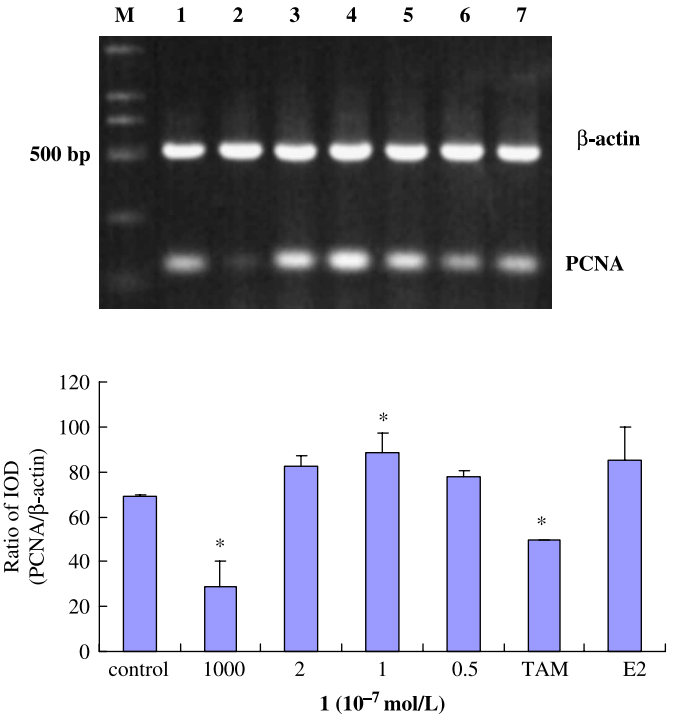


Figure 4. The effect of **1** on PCNA mRNA expression in MCF-7 cells was continuously exposed to **1** (1.0×10^{-4} and 0.5×10^{-7} –2.0×10^{-7} mol/l); mRNA expression determined by RT-PCR analysis as described under "Materials and Methods". 1, expression of control cells treated with 0.1% DMSO; 2, expression of cells treated with 1.0×10^{-4} mol/l **1**; 3–5, expression of cells treated with 0.5×10^{-7} –2.0×10^{-7} mol/l **1**; 6, expression of cells treated with 2.0×10^{-7} mol/l TAM; 7, expression of cells treated with 1.0 nmol/l estradiol.
Note: $n = 3$, compared with control group; $*p < 0.05$.

anti-rabbit IgG were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd, Beijing, China.

3.2 Test drugs

The test drug, resveratrol (**1**, >98% pure), was purchased from NICPBP (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). Estradiol and TAM were purchased from Sigma Chemical Co.

3.3 Cell culture

Human breast carcinoma MCF-7 cells, obtained from the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, were maintained in RPMI-1640 medium containing 10% heat-inactivated FBS. Four days before the addition of the test compound, the cells were transferred into phenol red-free RPMI-1640 medium containing 5% charcoal/dextran-treated FBS. Cells were maintained at 37°C under 5% CO_2 in a humidified incubator.

3.4 MTT assay

Cells were detached by trypsinization, harvested, counted, transferred into 96-well plates, and incubated for 24 h prior to treatment. The test samples were prepared by dissolving compounds in DMSO and followed by dilution with supplemented phenol red-free RPMI-1640 medium to yield a final concentration of test compounds at 0.5 and 1.0×10^{-4} mol/l and 0.5, 1.0, and

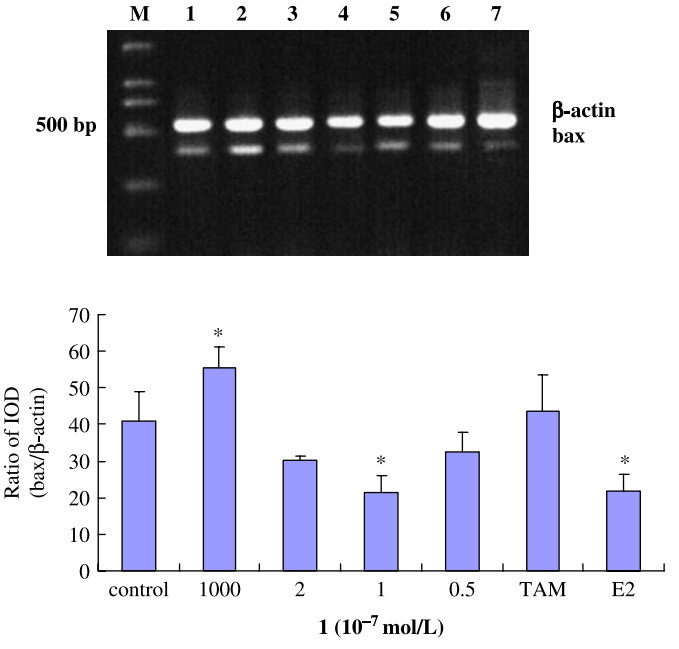


Figure 5. Effect of **1** on Bax mRNA expression in MCF-7 cells was continuously exposed to **1** (1.0×10^{-4} and 0.5×10^{-7}–2.0×10^{-7} mol/l), mRNA expression determined by RT-PCR analysis as described under "Materials and Methods". 1, expression of control cells treated with 0.1% DMSO; 2, expression of cells treated with 1.0×10^{-4} mol/l **1**; 3–5, expression of cells treated with 0.5×10^{-7}–2.0×10^{-7} mol/l **1**; 6, expression of cells treated with 2.0×10^{-7} mol/l TAM; 7, expression of cells treated with 1.0 nmol/l estradiol.
Note: $n = 3$, compared with control group; $*p < 0.05$.

2.0×10^{-7} mol/l. The final DMSO concentration in the assay was less than 0.1%. After incubation of test samples with MCF-7 cells (48 h), MTT solution (50 μl, 1 mg/ml) was added. After a 4-h incubation at 37°C, the medium was aspirated and the violet crystals were dissolved with 150 μl of DMSO. Then, the optical density was recorded using a micro-plate reader at 490 nm. E_2 (1.0×10^{-9} mol/l) and TAM (1.0×10^{-7} mol/l), dissolved in 0.1% DMSO, were used as the controls and 0.1% DMSO in phenol red-free RPMI-1640 media as solvent control. The assay was conducted in triplicate for each sample concentration, as well as the positive and solvent controls.

3.5 *Cellular growth curve control*

MCF-7 cells in log phase were trypsinized and seeded in 96-well plates at a density of 5000/ml. The cells were treated with different concentrations of **1** in phenol red-free RPMI-1640 media. The effects on cell growth were examined by MTT assay every 24 h for 7 successive days in order to complete the growth curve *in vitro*.

3.6 *Cell cycle analysis*

MCF-7 cells were plated at a density of 5000/ml cells in a 75 ml culture flask and incubated for 24 h. Fresh phenol red-free RPMI-1640 media containing various concentrations of **1** were added to culture flasks and incubated for 24 and 48 h, respectively. Cells were harvested, washed with PBS, and then fixed with 70% ethanol. The fixed cells were kept overnight at −20°C, washed with PBS before analysis, and then the propidium iodide solution (50 μg/ml propidium iodide in PBS, plus RNase, 50 μg/ml) was added.

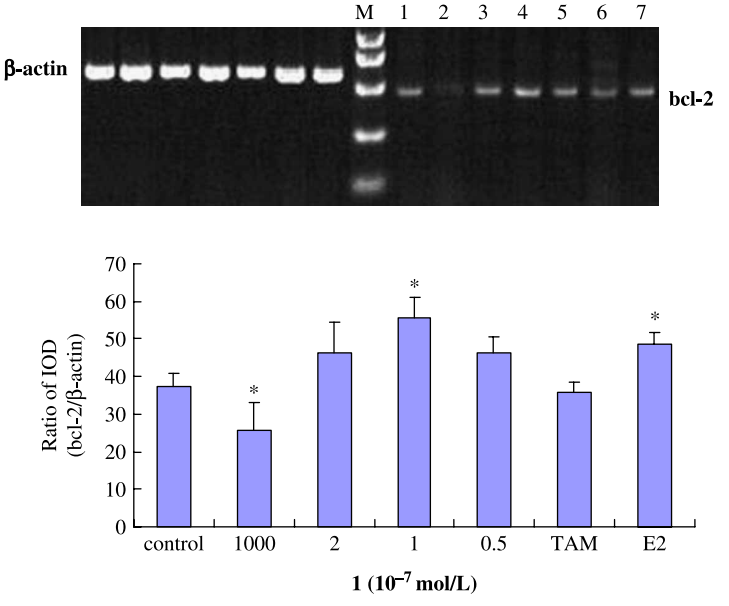


Figure 6. Effect of **1** on bcl-2 mRNA expression in MCF-7 cells was continuously exposed to **1** (1.0×10^{-4} and 0.5×10^{-7} –2.0×10^{-7} mol/l), mRNA expression determined by RT-PCR analysis as described under "Materials and Methods". 1, expression of control cells treated with 0.1% DMSO; 2, expression of cells treated with 1.0×10^{-4} mol/l **1**; 3–5, expression of cells treated with 0.5×10^{-7} –2.0×10^{-7} mol/l **1**; 6, expression of cells treated with 2.0×10^{-7} mol/l TAM; 7, expression of cells treated with 1.0 nmol/l estradiol.
Note: $n = 3$, compared with control group; $^{*}p < 0.05$.

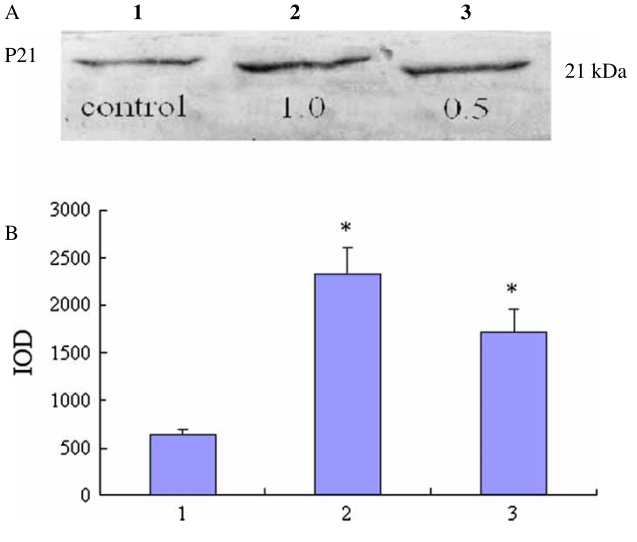


Figure 7. Effect of **1** on P21 protein expression in MCF-7 cells was continuously exposed to **1** (0.5×10^{-4} and 1.0×10^{-4} mol/l), protein expression determined by western blot analysis as described under "Materials and Methods". 1, expression of control cells treated with 0.1% DMSO; 2, expression of cells treated with 1.0×10^{-4} mol/l **1**; 3, expression of cells treated with 0.5×10^{-4} mol/l.
Note: $n = 3$, compared with control group; $^{*}p < 0.05$.

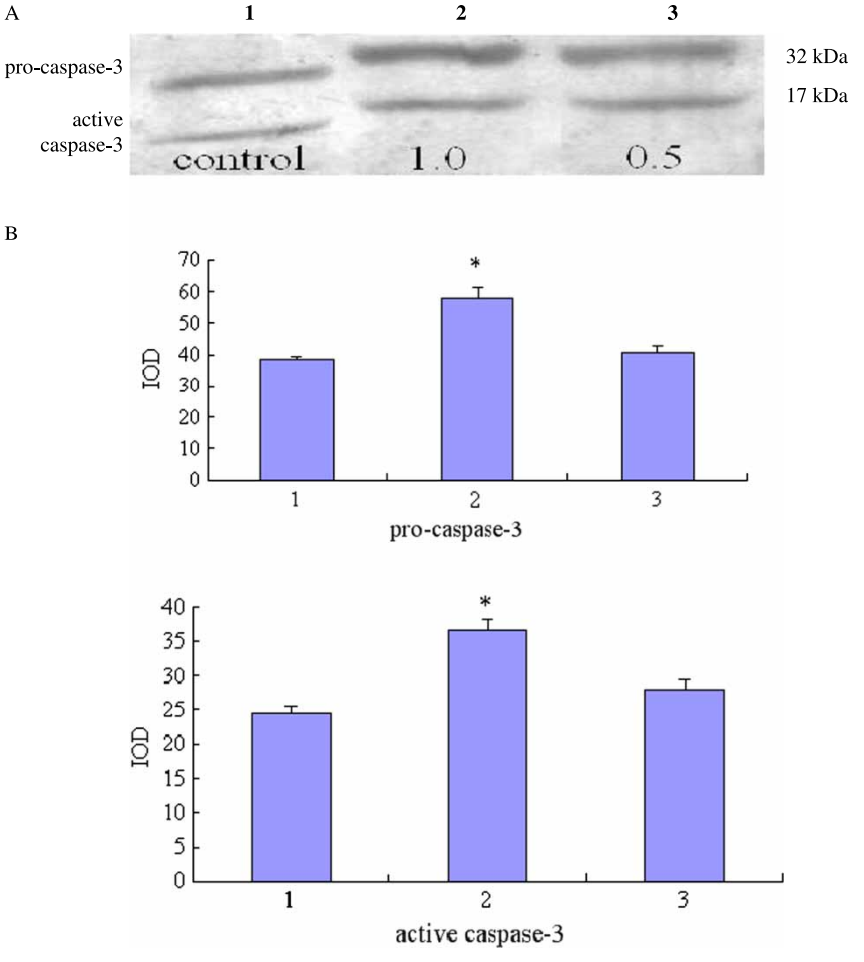


Figure 8. Effect of **1** on caspase-3 protein expression in MCF-7 cells was continuously exposed to **1** (0.5×10^{-4} and 1.0×10^{-4} mol/l), protein expression determined by western blot analysis as described under "Materials and Methods". 1, expression of control cells treated with 0.1% DMSO; 2, expression of cells treated with 1.0×10^{-4} mol/l **1**; 3, expression of cells treated with 0.5×10^{-4} mol/l.
Note: $n = 3$, compared with control group; $*p < 0.05$.

Cellular DNA content was analyzed by flow cytometry using a Coulter Epics-XL laser-based flow cytometer. At least 1.0×10^{-6} cells were used for each analysis, and results were displayed as histograms. PI (%) = $(S + G_2M)/(G_0/G_1 + S + G_2M) \times 100\%$.

3.7 Semi-quantitative RT-PCR

Total RNA was isolated from cells using Trizol reagent. After denaturing (94°C, 5 min), 500 ng of RNA was transcribed into cDNA, which was amplified using the following primers: PCNA 5′-GGA AAT GGA AAC ATT AAA TTG TCA C-3′, and 5′-GAG TGG CTT TTG TAA AGA AGT TCA G-3′; Bcl-2 5′-GGT GCC ACC TGT GGT CCA CCT-3′, and 5′-CTT CAC TTG TGG CCC AGA TAG G-3′; Bax 5′-CGT CCA CCA AGA AGC TGA GCG-3′, and 5′-AGC ACT CCC GCC ACA AAG ATG-3′. Duplex amplification was performed using a thermocycler for 28 cycles according to the following program: PCNA 30 s at 94°C, 30 s

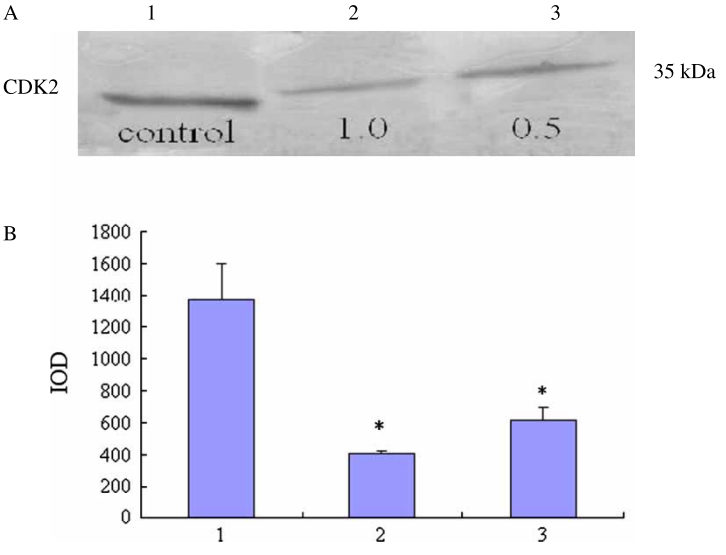


Figure 9. Effect of **1** on CDK2 protein expression in MCF-7 cells was continuously exposed to **1** (0.5×10^{-4} and 1.0×10^{-4} mol/l), protein expression determined by western blot analysis as described under "Materials and Methods". 1, expression of control cells treated with 0.1% DMSO; 2, expression of cells treated with 1.0×10^{-4} mol/l **1**; 3, expression of cells treated with 0.5×10^{-4} mol/l.
Note: $n = 3$, compared with control group; $*p < 0.05$.

at 56°C, and 30 s at 72°C; Bcl-2 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C; Bax 1 min at 94°C, 45 s at 58°C, and 30 s at 72°C. The final products (133, 458 and 382 bp, respectively) were confirmed on a 1% agarose gel. Sets of primers for β-actin were used as a positive internal control.

3.8 *Western blot analysis*

MCF-7 cells were exposed to various concentrations of **1** for 48 h. Then, the cells were lysed in 50 mM tris (pH 7.4), 120 mM NaCl, and 0.5% Nonidet P-40 supplemented with 2 μg/ml aprotinin and 100 μg/ml phenylmethylsulfonyl fluoride. The lysate

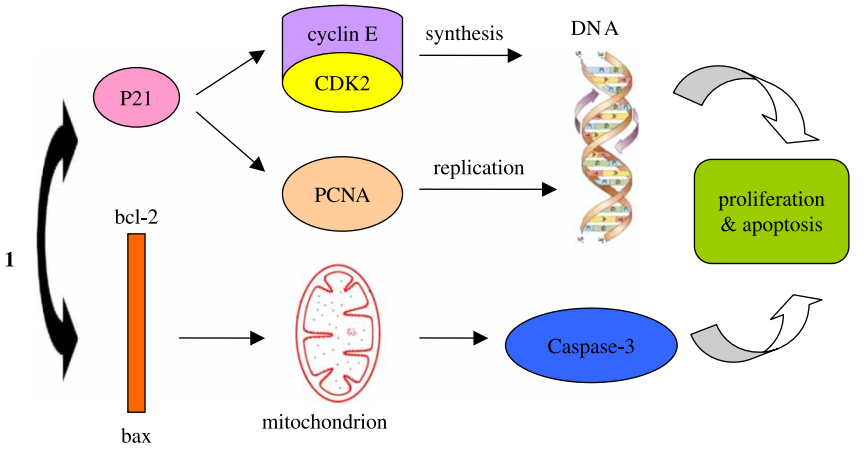


Figure 10. Schematic diagram of the effect of **1** on MCF-7 cells.

was cleared by centrifugation, quantified, and boiled for 5 min in Laemmli buffer (50 mM tris (pH 6.8), 1% of 2-mercaptoethanol, 2% of SDS, 0.1% of bromophenol blue, and 10% of glycerol). Proteins were separated by SDS-12 or 15% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Membranes were probed with anti-p21 antibodies (1:100), anti-CDK2 antibodies (1:100), and anti-caspase-3 (1:100). Antibodies were diluted in tris-buffered saline plus 0.1% Tween 20 plus 5% milk. Membranes were then incubated in the appropriate secondary antibody before being visualized by DAB. The results of western blot were captured by quantitative analysis, which the band intensities were quantified from scanned images using Gel-Pro Analyzer 3.1 Automated Digitizing System Software.

3.9 Statistical analysis

Values are presented as means ± SD. Group means were compared by ANOVA using Fisher's *post hoc* analysis with Excel Software (Microsoft). $p < 0.05$ was considered significant. Figure data are representative of at least three independent experiments.

Acknowledgements

The authors gratefully thank the Science Foundation of Chinese People's Armed Police Forces (WBS2007-12) for financial support of this research.

References

[1] H. Adlercreutz, *J. Steroid Biochem. Mol. Biol.* **83**, 113 (2002).
[2] O. JE, A. GL, P. RL. *JAMA* **288**, 321 (2002).
[3] Q.S. Meng, P. He, and X.Y. Zhu, *Chem. Life* **27**, 141 (2007).
[4] X.J. Zhang, Y. Zou, and R.Q. Zhong, *Chin. New Drugs, J.* **13**, 692 (2004).
[5] L. Shen, D. Zhang, and L. Zhang, *Med. Recapit.* **9**, 565 (2003).
[6] S. Tommasi and G.P. Pfeifer, *J. Biol. Chem.* **274**, 27829 (1999).
[7] W.W. Wang, E.D. Smith, and J.E. Stuff, *Am. J. Clin.* **68**, 1385 (1998).
[8] G.X. Gu, *J. Clin. Rehabil. Tissue Eng. Res.* **11**, 5566 (2007).
[9] H. Abe, M.A. Shibata, and Y. Otsuki, *Mol. Hum. Reprod.* **12**, 535 (2006).
[10] M. Spreafico, E. Boriani, and M. Novic, *Mol. Divers.* **11**, 171 (2007).
[11] M. Alkhalaf, A. El-Mowafy, and W. Renno, *Arch. Med. Res.* **39**, 162 (2008).
[12] U. Sunil, N. Madhavi, and R. Sreenivasa, *Clin. Cancer Res.* **7**, 1782 (2001).