Arch Pharm Res Vol 28, No 1, 68-72, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Pseudolaric Acid B Induces Apoptosis Through p53 and Bax/Bcl-2 Pathways in Human Melanoma A375-S2 Cells

Xian-Feng Gong[1,2], Min-Wei Wang[2], Shin-Ichi Tashiro[3], Satoshi Onodera[3], and Takashi Ikejima[1]

[1]China-Japan Research Institute of Medical and Pharmaceutical Sciences, [2]Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang 110016, China and [3]Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo 194-8543, Japan

(Received July 5, 2004)

Pseudolaric acid B is a major compound found in the bark of *Pseudolarix kaempferi Gordon*. In our study, pseudolaric acid B inhibited growth of human melanoma cells, A375-S2 in a time- and dose-dependent manner. A375-S2 cells treated with pseudolaric acid B showed typical characteristics of apoptosis including morphologic changes, DNA fragmentation, sub-diploid peak in flow cytometry, cleavage of poly-ADP ribose polymerase (PARP) and degradation of inhibitor of caspase-activated DNase (ICAD). P53 protein expression was upregulated while cells were arrested at the G_2/M phase of the cell cycle. There was a decrease in the expression of anti-apoptotic Bcl-2 and Bcl-xL proteins, whereas pro-apoptotic Bax was increased. The two classical caspase substrates, PARP and ICAD, were both decreased in a time-dependent manner, indicating the activation of downstream caspases.

Key words: Pseudolaric acid B, Apoptosis, A375-S2

INTRODUCTION

Pseudolaric acid B (Fig. 1), a novel diterpene acid isolated from the bark of *Pseudolarix kaempferi Gordon*. (Pinaceae), has been widely used for the treatment of fungal infections. It exerts potent antifungal, antimicrobial (Li *et al.*, 1995), antifertility (Wang *et al.*, 1982; Wang *et al.*, 1988) and cytotoxic activity (pan *et al.*, 1990). Recent reports show that pseudolaric acid B can induce cell apoptosis, however, its mechanism remains unclear.

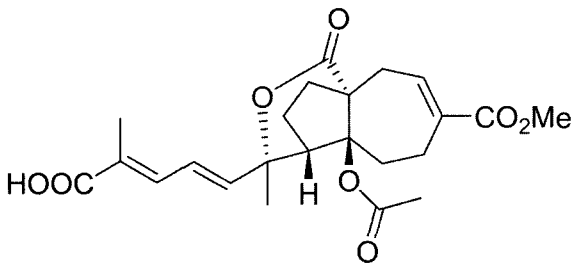

Fig. 1. The chemical structure of pseudolaric acid B

Correspondence to: Ikejima Takashi, China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Shenyang 110016, China
Tel/Fax: 86-24-23844463
E-mail: ikejimat@vip.sina.com

Apoptosis, defined as programmed cell death, plays an important role in many physiological processes such as embryo and organ development, immune response and tumor development and growth. Detecting apoptotic cells or monitoring cells progressing to apoptosis are essential steps in basic research for the development of drugs that may regulate apoptosis (Yue *et al.*, 1999). A set of morphologic and biochemical features characterize apoptosis. Chromatin condensation, cell shrinkage, membrane blebbing, and apoptotic body formation are changes that typically occur in this sequential order (Kerr *et al.*, 1972).

In this study, we investigated the apoptotic effect of pseudolaric acid B on human melanoma A375-S2 cells.

MATERIALS AND METHODS

Chemical reagents

Pseudolaric acid B was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Hoechst 33258, RNase A, Proteinase K, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). Rabbit polyclonal

antibodies against poly-ADP ribose polymerase (PARP) and inhibitor of caspase- activated DNase (ICAD), mouse monoclonal antibodies against Bcl-2, Bcl-xL, and p53, as well as horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit and goat-anti-mouse) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against Bax was obtained from Oncogene Research Products (Cambridge, MA).

Cell culture

A375-S2, human melanoma cells, were obtained from American Type Culture Collection (ATCC, #CRL, 1872, MD, USA) and were cultured in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% heat inactivated (56 °C, 30 min) fetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 $mmolL^{-1}$ L-glutamine (Gibco, Grand Island, NY), containing 5% CO_2 at 37 °C.

Cell growth inhibition test

Cell growth was measured by MTT test. After incubation with pseudolaric acid B, A375-S2 cells (5×10^4/well) in a 96-well plate were washed once with PBS and MTT (500 mg/mL in RPMI-1640 medium) was added to each well. The cells were further incubated at 37 °C for 4 h, and DMSO (0.15 mL) was added to dissolve the formazan crystals. Absorbance was measured at 492 nm with enzyme-linked immunosorbent assay plate reader (Tecan, Salzburg, Austria).

Observation of morphologic changes by light microscopy

A375-S2 cells were treated with pseudolaric acid B (5 μM) for 36 h. Morphologic changes were observed by phase contrast microscopy (Leica, Wetzlar, Germany).

Nuclear damage observed by Hoechst 33258 staining

After treatment with pseudolaric acid B, A375-S2 cells were collected by centrifugation at 1,000 × g for 5 min, and washed twice with PBS. The cells were fixed with 3.7% paraformaldelyde at room temperature for 2 h, centrifuged, washed with PBS, stained with Hoechst 33258, fixed on glass slides, and then observed with fluorescence microscopy (Nikon, Tokyo, Japan).

Detection of DNA fragmentation by agarose gel electrophoresis

For DNA extraction, both adherent and floating A375-S2 cells were collected by centrifugation at 1000 × g for 5 min. The cell pellet was suspended in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, pH 8.0, and 0.5% Triton-X 100) and kept at 4 °C for 10 min. The lysate was centrifuged at 25 000 × g for 20 min. The supernatant was incubated with RNase A (40 ng/mL) at 37 °C for 1 h, and then incubated with proteinase K (40 ng/mL) at 37 °C for 1 h. The supernatant was mixed with 0.5 M NaCl and 50% 2-propanol overnight at -20 °C, then centrifuged at 25,000 × g for 15 min. After drying, DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, pH 8.0) and separated by 2% agarose gel electrophoresis at 100 V for 50 min.

Flow cytometric analysis

After exposure to pseudolaric acid B, the cells were harvested, washed twice with PBS and fixed with cold 70% ethanol at 4 °C overnight. Fixed cells were resuspended in 1 mL of propidium iodide (PI) solution (0.1 mg/mL PI, 1 mg/mL sodium citrate, 0.3% Triton-X 100, and 0.05 mg/mL RNase) at room temperature for 30 min and analyzed by FACS Calibur flow cytometry (BD, Franklin Lakes, NJ, USA).

Western blot analysis of protein expression

A375-S2 cells were treated with 5 μM pseudolaric acid B for 0, 12, 24, 36, and 48 h. Both adherent and floating cells were collected and frozen at -80 °C. Western blot analysis was performed as previously described with minor modifications (Wu *et al.*, 2003). The cell pellets were resuspended in lysis buffer, including 50 mM Hepes, pH 7.4, 1% Triton-X 100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 M PMSF, 0.1 mg/mL aprotinin, and 0.01 mg/mL leupeptin, and then lysed in 4 °C for 1 h. After centrifugation at 13,000 × g for 10 min, the protein content of the supernatant was determined using the Bio-Rad protein assay reagent (Bio-Rad, USA). The protein lysates were separated by electrophoresis in 12% SDS polyacrylamide gel and blotted onto nitrocellulose membrane. Protein expression was detected using primary polyclonal antibody and secondary polyclonal antibody conjugated with peroxidase.

RESULTS

Inhibitory effects of pseudolaric acid B on cell growth

Pseudolaric acid B inhibited A375-S2 cell growth in a time- and dose-dependent manner. Treatment with pseudolaric acid B (0.16-500 μM) showed an increasing growth inhibition on A375-S2 cells (Fig. 2). After 36 h treatment with pseudolaric acid B, we observed a decrease in the total number of cells and an accumulation of cells floating in the culture medium, indicating pseudolaric acid B-induced cell death (Fig. 3B).

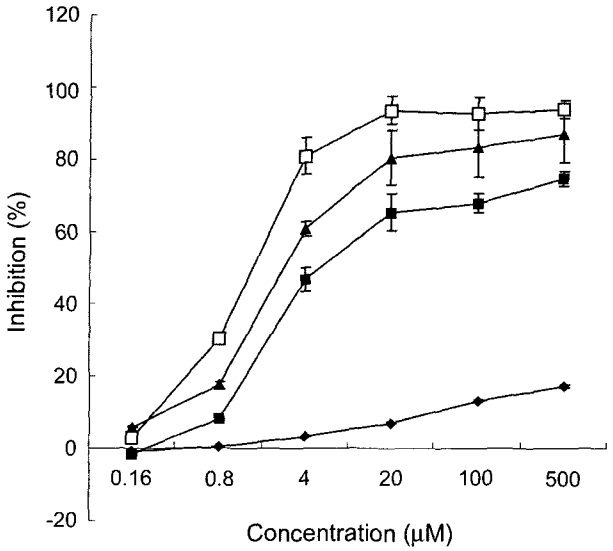

Fig. 2. Inhibitory effects of pseudolaric acid B on A375-S2 cell growth. The cells (5×10^4 cells per well) were incubated with various concentrations of pseudolaric acid B for 12 h (◆), 24 h (■), 36 h (▲), or 48 h (□). Growth inhibition was evaluated by the MTT method.

Pseudolaric acid B-induced morphologic changes and DNA fragmentation

Nuclear morphological changes were observed by Hoechst 33258 staining. In the control sample, A375-S2 cells were round in shape and stained homogeneously (Fig. 3C). After 36 h treatment with pseudolaric acid B, blebbing nuclei and granular apoptotic bodies appeared (Fig. 3D, arrows). In A375-S2 cells incubated with pseudolaric acid B (5 μM) for 12, 24, 36 h or with pseudolaric acid B (0, 2.5, 5, 10 μM) for 36 h, typical DNA fragmentation was observed indicative of apoptosis (Fig. 4).

Pseudolaric acid B-induced G_2/M arrest and upregulation of p53

To investigate further features of cell growth inhibition by pseudolaric acid B, flow cytometric analysis was performed. After treatment with increasing concentrations of pseudolaric acid B for 24 h, the cells had accumulated in the G_2/M phase. Sub-G_0/G_1 peak was also observed (Fig. 5). Simultaneously, p53 protein had also accumulated at 24 h (Fig. 6).

Effects of pseudolaric acid B on the expression of Bcl-2, Bcl-xL and Bax

Since anti- and pro-apoptotic members of the Bcl-2 family determine life or death for the cell, we measured the expressions of Bcl-2, Bcl-xL, and Bax by Western blot analysis. After incubation with pseudolaric acid B, expression of Bcl-2 and Bcl-xL had decreased; in contrast, Bax protein level increased in a time-dependent manner (Fig. 7).

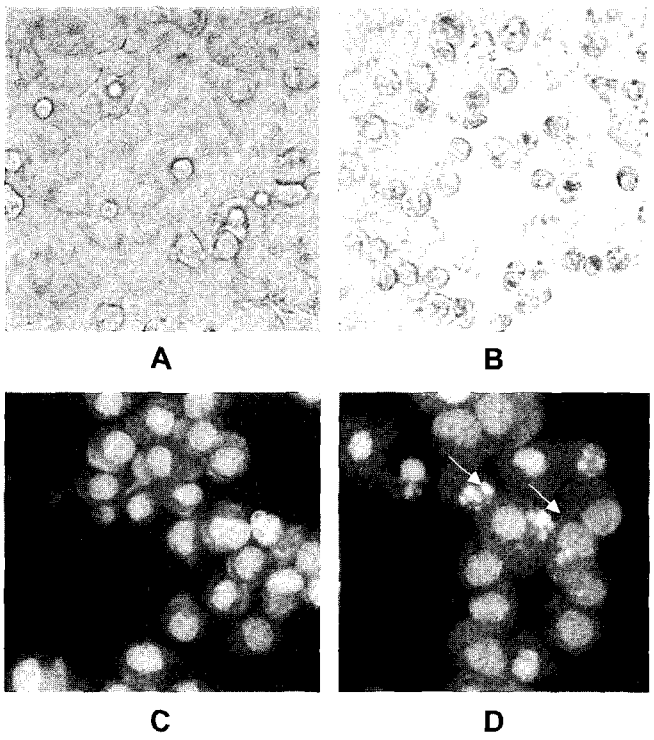

Fig. 3. Pseudolaric acid B induced morphologic changes of A375-S2 cells by photomicroscope (A and B) and fluorescence microscope (C and D). A and C: control. B and D: the cells were treated with 5 μM pseudolaric acid B for 36 h. Arrows indicate the condensed nuclei.

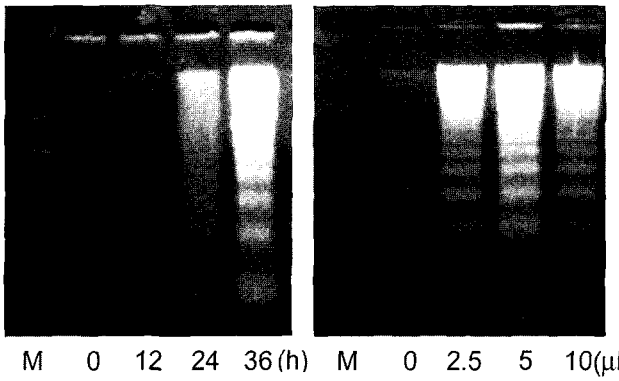

Fig. 4. Pseudolaric acid B induced DNA fragmentation in A375-S2 cells. The cells were cultured in the presence of 5 μM pseudolaric acid B at varying times (left) or varying concentrations of pseudolaric acid B for 36 h (right). Lane M: DNA molecular weight marker.

Effects of pseudolaric acid B on the expression of PARP and ICAD

Since PARP and ICAD were classical substrates of caspases, we measured the expression of PARP and ICAD by Western blot analysis. The results showed that both PARP and ICAD expression had decreased,

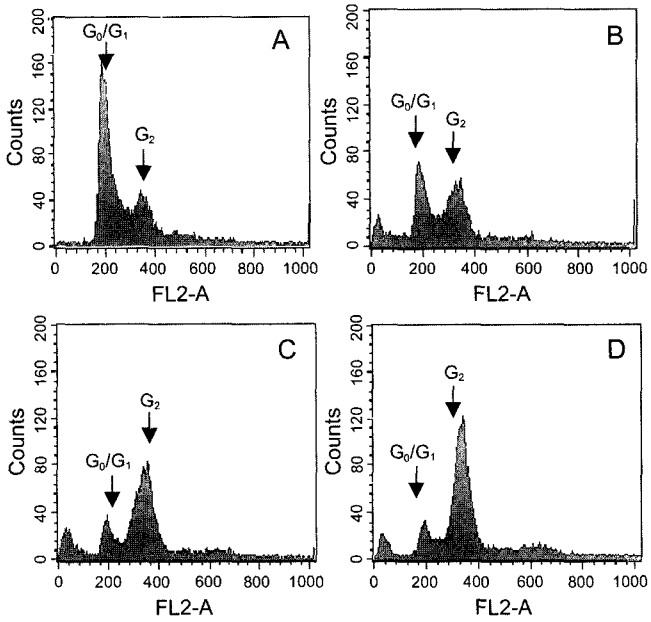

Fig. 5. G_2/M arrest induced by pseudolaric acid B in A375-S2 cells. The cells were cultured in the presence of varying concentrations of pseudolaric acid B for 24 h. Sub-G_0/G_1 peak was observed in pseudolaric acid B-treated cells. A, B, C, and D indicate cells treated with pseudolaric acid B (0, 0.5, 1, and 5 μM), respectively.

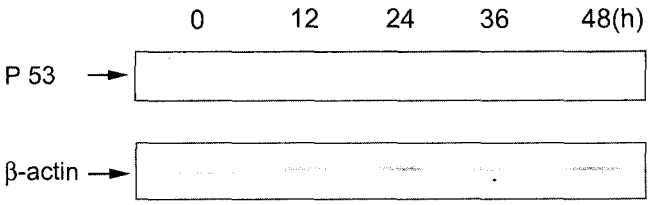

Fig. 6. The expression of p53 in the presence of pseudolaric acid B (5 μM) at varying times in A375-S2 cells. Western blot analyses were performed with anti-p53 or β-actin antibody.

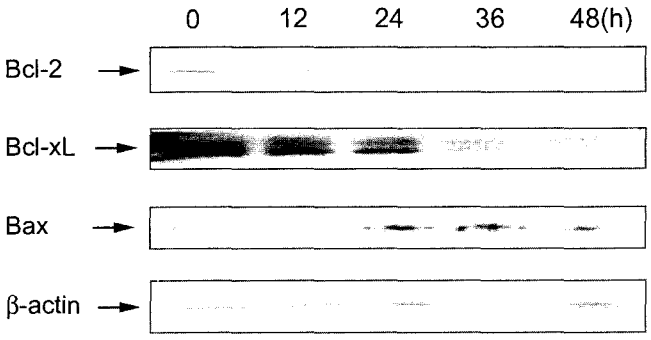

Fig. 7. The expression of Bcl-2, Bcl-xL and Bax in the presence of pseudolaric acid B (5 μM) at varying times in A375-S2 cells. Western blot analyses were performed with anti-Bcl-2, Bcl-xL, Bax, or β-actin antibody.

suggesting that caspases participated in pseudolaric acid B induced A375-S2 cell death (Fig. 8).

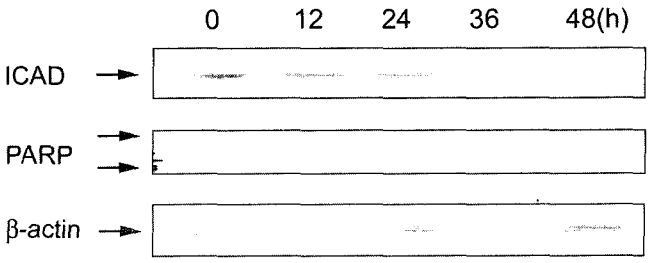

Fig. 8. The expression of ICAD and PARP in the presence of pseudolaric acid B (5 μM) at varying times in A375-S2 cells. Western blot analyses were performed with anti-ICAD, PARP, or β-actin antibody.

DISCUSSION

Pseudolaric acid B is isolated from Tu-Jin-Pi, a traditional chinese medicine used for the treatment of dermatological fungal infections. To date, little has been reported about the anti-tumor mechanism of pseudolaric acid B. In this study, we examined antiproliferative effects in A375-S2 cells and further studied the molecular basis of anti-oncogenic properties of pseudolaric acid B on the cells.

The tumor suppressor gene, *p53*, is involved in the regulation of cell cycle, DNA repair and activation of apoptosis. In response to DNA damage, cells exhibit a rapid increase in wild-type p53 protein levels and temporarily arrest in the G_1 phase (Kastan *et al.*, 1991). Furthermore p53 was suggested to play a role in the regulation of cell cycle arrest at G_2/M (Stewart *et al.*, 1995; Schwartz *et al.*, 1997; Hermeking *et al.*, 1997; Taylor and Stark, 2001). When optimal repair after DNA damage is impossible, p53 plays a major role in apoptosis (Lane, 1992).

It is plausible that the observed G_2/M cell cycle arrest induced by pseudolaric acid B was causally related to the up-regulation of p53. Using a combination of flow cytometry and Western blot analysis, we demonstrated that the time of G_2/M arrest closely paralleled elevated levels of p53 and was observed 24 h after treatment with 5 μM pseudolaric acid B.

Bcl-2 is present in the inner membrane of the nucleus, mitochondria, and endoplasmic reticulum (Hockenbery *et al.*, 1991) and is known to form a homo- or hetero-dimer with its counter-protein, Bax, the ratio of Bcl-2 and Bax eventually decides whether the cell follows the apoptotic pathway (Agarwal and Mehta, 1997; Mengubas *et al.*, 1996).

A375-S2 cells treated with pseudolaric acid B exhibited elevated levels of pro-apoptotic Bax expression, while anti-apoptotic Bcl-2 was down-regulated. These results suggest that the mitochondrial pathway of cell death might be involved in pseudolaric acid B-induced A375-

S2 cell death.

PARP is a nuclear enzyme involved in DNA repair processes. During apoptosis, the PARP protein is cleaved into 24 kDa and 85 kDa fragments by caspase-3 activity (Nicholson *et al.*, 1995; Lazebnik *et al.*, 1994). Caspase-activated DNase (CAD) exists as an inactive complex with ICAD. Caspase-3 activated by apoptotic signals cleaves ICAD to release CAD (Sakahira *et al.*, 1998), which then enters the nucleus to degrade chromosomal DNA (Enari *et al.*, 1998; Liu *et al.*, 1997). The specific proteolytic cleavage of PARP and ICAD is considered to be hallmarks of apoptosis. Western blot analysis showed that PARP is cleaved to 85 kDa and ICAD is degraded, indicating that caspase-3 was activated.

In conclusion, the caspase cascade and regulation of Bax, Bcl-2, Bcl-xL and p53 were involved in pseudolaric acid B-induced A375-S2 cell apoptosis. More detailed mechanisms of pseudolaric acid B-induced A375-S2 cell apoptosis remains to be elucidated.

REFERENCES

Agarwal, N. and Mehta, K., Possible involvement of Bcl-2 pathway in retinoid X receptor alpha-induced apoptosis of HL-60 cells. *Biochem. Biophys. Res. Comm.*, 230, 251-253 (1997).

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S., A., Caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*, 391, 43-50 (1998).

Hermeking, H., Lengauer, C., Polyak, K., He, T. C., Zhang, L., Thiagalingam, S., Kinzler, K. W., and Vogelstein, B., 14-3-3-Sigma is a p53-regulated inhibitor of G_2/M progression. *Mol. Cell*, 1, 3-11 (1997).

Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., and Korsmeyer, S. J., Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, 75, 241-251 (1993).

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W., Participation of p53 protein in cellular response to DNA damage. *Cancer Res.*, 51, 6304-6311 (1991).

Kerr, J.F., Wyllie, A.H., and Currie, A.R., Apoptosis: a basic biologic phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, 26, 239-257 (1972).

Lane, D.P., p53, guardian of the genome. *Nature*, 258, 15-16 (1992).

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C., Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*, 371, 346-347 (1994).

Li, E., Clark, A. M., and Hufford, C. D., Antifungal evaluation of pseudolaric acid B, a major constituent of *pseudolarix kaempferi*. *J. Nat. Prod.*, 58, 57-67 (1995).

Liu, X. S., Zou, H., Slaughter, C., and Wang, X., DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, 89, 175-184 (1997).

Mengubas, K., Riordan, F. A., Hoffbrand, V., and Wickremasinghe, G., Co-ordinated downregulation of Bcl-2 and Bax expression during granulocytic and macrophage-like differentiation of the HL60 premyelocytic leukemia cell line. *FEBS. Lett.*, 394, 356-360 (1996).

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., and Lazebnik, Y. A., Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, 376, 37-43 (1995).

Pan, D. J., Li, Z. L., Hu, C. Q., Chen, K., Chang, J. J., and Lee, K. H., The cytotoxic principles of *Pseudolarix Kaempferi*: Pseudolaric acid-A and B and related derivatives. *Planta Med.*, 56, 383-385 (1990).

Sakahira, H., Enari, M., and Nagata, S., Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*, 391, 96-99 (1998).

Schwartz, D., Almong, N., Peled, A., Goldfinger, N., and Rotter, V., Role of wild-type p53 in the G_2 phase: regulation of the α-irradiation induced delay and DNA repair. *Oncogene*, 15, 2597-2607 (1997).

Stewart, N., Hicks, G. G., Paraskevans, F., and Mowat, M., Evidence for a second cell cycle block at G_2/M by p53. *Oncogene*, 10, 109-115 (1995).

Taylor, W.R., and Stark, G.R., Regulation of the G_2/M transition by p53. *Oncogene*, 20, 1803-1815 (2001).

Wang, W. C., Lu, R.F., Zhao, S. X., and Gu, Z. P., Comparison of early pregnancy-terminating effect and toxicity between pseudolaric acids A and B. *Acta. Pharmacol. Sin.*, 9, 445-448 (1988).

Wang, W. C., Lu, R. F., Zhao, S. X., and Zhu, Y. Z., Antifertility effect of pseudolaric acid B. *Acta. Pharmacol. Sin.*, 3, 188-192 (1982).

Wu, L. X., Xu, J. H., Wu, G. H., and Chen, Y. Z., Inhibitory effect of curcumin on proliferation of K562 cells involves down-regulation of $p210^{bcr/abl}$-initiated Ras signal transduction pathway. *Acta. Pharmacol. Sin.*, 11, 1155-1160 (2003).

Yue, T. L., Ohlstein, H. E., and Ruffolo, R. R., Apoptosis: a potential target for discovering novel therapies for cardiovascular diseases. *Curr. Opin. Chem. Biol.*, 3, 474-480 (1999).