in vivo *21*: 1011-1020 (2007)

Shikonin-induced Apoptosis Involves Caspase-3 Activity in a Human Bladder Cancer Cell Line (T24)

CHIN-CHUNG YEH[1], HSIU-MAAN KUO[2], TE-MAO LI[3], JING-PIN LIN[4], FU-SHUN YU[5], HSU-FENG LU[6], JING-GUNG CHUNG[6,7] and JAI-SING YANG[8]

[1]Department of Urology, China Medical University Hospital, Taichung, Taiwan; Departments of [2]Parasitology, [6]Microbiology, [7]Biological Science and Technology, and [8]Pharmacology, [3]Graduate Institute of Chinese Medical Science, Schools of [4]Chinese Medicine and [5]Dentisty, China Medical University, Taichung,Taiwan; [6]Department of Clinical Pathology, Cheng Hsin Rehabilitation Medical Center, Taipei, Taiwan, R.O.C.

Abstract. *Apoptosis is a process that leads to programmed cell death and also a therapeutic target of cancer. In this study, potential apoptotic effects of shikonin on human bladder cancer cells (T24) in vitro were evaluated. Apoptosis induction, cell viability and morphological changes were investigated and caspase-3 and -9 activity was determined by flow cytometric assay and reverse transcription-polymerase chain reaction. The results showed marked differences in G0/G1 cell cycle arrest and cell death of the T24 cells between shikonin treated and untreated groups. Within 72 hours of treatment, shikonin influenced the cyclin dependent kinase (CDK) and cyclin activity by increasing p21 and decreasing cyclin E, CDK2 and CDK4 protein levels. A marked increase was found in apoptosis induction when the T24 cells were treated with shikonin compared to the untreated group, also confirmed by flow cytometry assay. Shikonin also promoted caspase-3 activity, which led to the induction of caspase-activated DNase (CAD) and cleavage poly(ADP-ribose)polymerase. Furthermore, the shikonin-induced apoptosis of the T24 cells was markedly blocked by the broad-spectrum caspase inhibitor, z-VAD-fmk. Shikonin may be a potential agent for the treatment of bladder transitional cell carcinoma since it induces apoptosis through the activation of caspase-3 activity in T24 human bladder cancer cells.*

Correspondence to: Dr. Jing-Gung Chung, Department of Microbiology, School of Biological Science and Technology, China Medical University, No 91, Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C. Tel: +886422053366-2161, Fax: +886422053764, e-mail: jgchung@mail.cmu.edu.tw / Dr. Jai-Sing Yang, Department of Pharmacology, China Medical University, No 91, Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C. Tel: +8864220533662225, Fax: +886422053764, e-mail: jaising@mail.cmu.edu.tw

Key Words: Shikonin, apoptosis, caspase-3, human bladder cancer, Western blotting, caspase-activated DNase.

The "People Health Bureau of Taiwan" has reported that about 3.47 persons per 100,000 people die per year of bladder cancer. Currently therapeutic approaches for human bladder cancer include radiotherapy, chemotherapy and surgery. However, the conventional strategies for treatment of human bladder cancer are not yet satisfactory. Many compounds purified from plants have revealed anticancer activity. It is well known that chemotherapeutic agents, such as taxol (1) and camptothecin (2) can induce cancer cells to differentiate and undergo apoptosis. Some anticancer drugs are known to induce apoptosis *via* the inhibition of topoisomerase II (2-5). Apoptosis is characterized by shrinkage of the cell, DNA fragmentation, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighbouring cells (6). Shikonin has been isolated from the root of *Lithospermum erythrorhizon* and others, and has shown anti-inflammatory activity (7) and inhibition of leukotriene B4 biosynthesis activity (8). Shikonin has been reported as a new potent inhibitor of topoisomerase I (9) and as an inhibitor of proliferation and migration of endothelial cells in culture and network formation by endothelial cells on Matrigel *in vitro* (10). It has also been demonstrated that shikonin induced apoptosis in the HL-60 human premyelocytic leukemia cell line (11) and that it is a novel inhibitor of cyclooxygenase-2 transcription (12). Gaddipati *et al.* (13) demonstrated the inhibitory effect of shikonin on cellular growth and insulin-like growth factors, especially in PC-3 cells and suggested potential for the treatment of prostate cancer. However, no information is currently available on the effects of shikonin on human bladder cancer cells. Thus, the present study was performed to determine whether or not shikonin could induce apoptosis and cell cycle arrest in human bladder cancer T24 cells.

Materials and Methods

Chemicals and reagents. Shikonin was obtained from Ichimaru Pharcos Co. Ltd. (Japan). Trypan blue, Tris-HCl, triton X-100, propidium iodide (PI), ribonuclease-A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates, and TE buffer were purchased from Merck Co. (Darmstadt, Germany). McCoy's 5a medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The Caspase-3 Assay Kit was bought from Boehringer Mannheim (Mannhein, Germany). The quality of all chemicals was reagent grade.

Human bladder cancer cell line (T24). The human bladder cancer cell line (T24) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C under humidified 5% CO_2 and 95% air at one atmosphere in McCoy's 5a medium supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% glutamine.

Cell viability determination. The cells were plated in 12 well plates at a density of $5x10^5$ cells/well and grown for 24 h. Different concentrations of shikonin were then added, while only DMSO (solvent) was added for the control regimen and grown at 37˚C, 5% CO_2 and 95% air for different periods of time. For determining cell viability, the trypan blue exclusion protocol was used. Briefly, about 10 μl of cell suspension in PBS was mixed with 40 μl of trypan blue, and the number of stained (dead) and unstained (live) cells were counted using a hemocytometer (14).

Cell morphology. The cells (T24) were plated in 12 well plates at a density of $1x10^5$ cells/well and grown for 24 h. Different concentrations of shikonin were added and they were grown at 37˚C in a humidified 5% CO_2 95% air for 24 h. For the cell morphology experiment, the culture plates were examined under a phase contrast microscope and photographed.

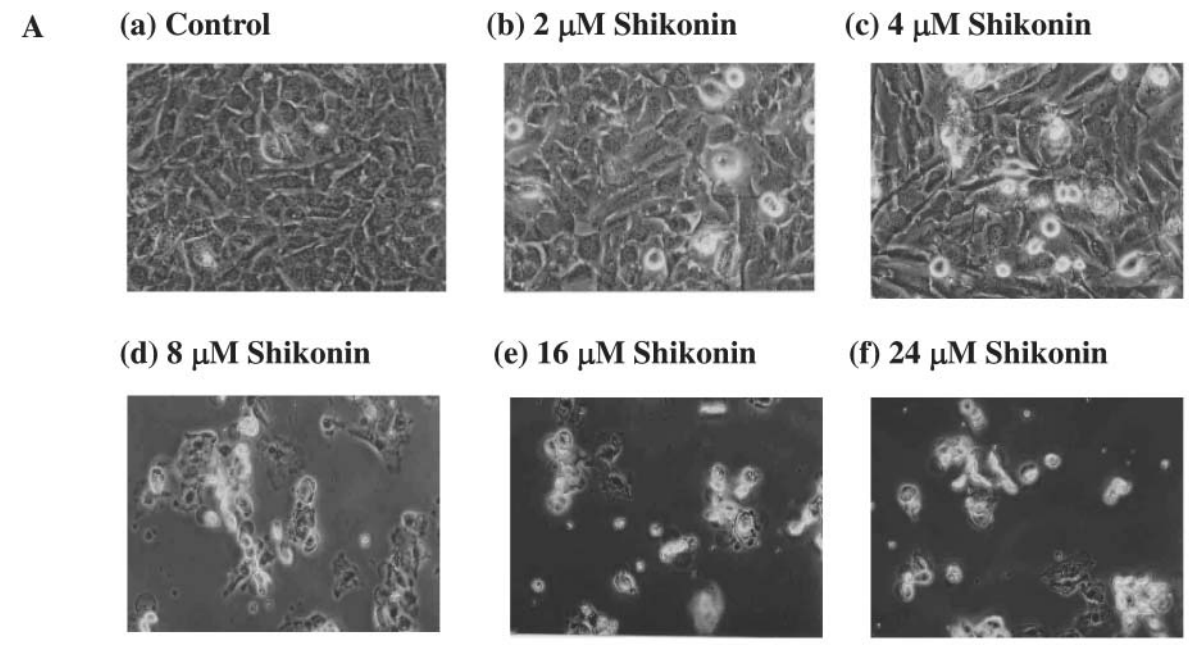
Flow cytometry analysis. To estimate the proportion of the T24 cells in different phases of the cell cycle and the apoptotic effect of shikonin, the cell DNA contents were measured using flow cytometry as described by Ormerod (15-16). About $5x10^5$ cells/well in 6-well plates with (0-16 μM) shikonin were incubated for different time periods, and then the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) with 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase (Sigma). After 30 minutes at 37˚C, the cells were analyzed with a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argonion laser at 488 nm wave-length and the cell cycle was determined.

Poly(ADP-ribose)polymerase (PARP) monoclonal antibody assay. The cells were plated in 12 well plates at a density of $5x10^5$ cells/well and grown for 24 h. Then various concentrations of shikonin were added, while only DMSO (solvent) was added for the control regimen, and the cells were grown at 37˚C in a humidified 5% CO_2 for 12 h. The PARP monoclonal antibody assay (Alexis, San Diego, CA, USA) was applied. After centrifugation, the medium was discarded and the cells were washed twice with PBS. The supernatant was removed and the cells were resuspended in the remaining 100-200 μl PBS and then transferred to 96 well dishes. Subsequently the dishes were centrifuged at 1500 rpm for 4 minutes at 4˚C, and the supernatant was removed and the cells were fixed in 100 μl of 3.7% formaldehyde at 4˚C for 15 minutes. They were washed again with PBS, and centrifuged to remove the supernatant and 100 μl of 0.2% NP-40 were added for 15 minutes. After centrifugation to remove the supernatant, PBS with 5% of non-fat milk formaldehyde containing 1% PARP monoclonal antibody was added and the cells were left at 4˚C overnight. Finally the supernatant was removed. Fifty μl 1% FITC-conjugated goat anti-mouse IgG antibody (secondary antibody) was added for 30 minutes in the dark and following washing with PBS the cells were observed under a fluorescence microscope and photographed.

Caspase-3 activity determination. About $5x10^6$ cells (T24) were lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 1 mM PMSF, 1 μg/ml aprotinin, 1 mg/ml leupeptin) for 30 minutes at 4˚C followed by centrifugation at 10,000 xg for 30 minutes. For caspase-3 activity determination, 50 μl caspase-3 specific reaction mixture was used. The substrate peptide (200 μM) was incubated at 37˚C with cytosolic extracts (15 μg of total protein) from T24 cells after incubation with or without shikonin in reaction buffer (100 mM HEPES, 10% sucrose, 10 mM DTT, 0.1% 3-[3-chloamidopropyl] dimethylammonio) 1-propanesulfonate. Fluorescence was determined after 2 h (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a fluorescence plate reader (Fluoroskan Ascent, Labsystems).

Inhibition of shikonin-induced apoptosis by the caspase inhibitor (z-VAD-fmk). The T24 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor z-VAD-fmk 3 hours prior to treatment with shikonin. Then apoptosis and caspase activity were determined as described above.

Reverse transcriptase-polymerase chain reaction (RT-PCR). The total RNA was extracted from the T24 cells using a Qiagen RNeasy Mini Kit, as described previously (17), 24 h after cotreatment with or without different concentrations of shikonin. Total RNA (1.5 μg), 0.5 μg of oligo-dT primer and DEPC (diethyl pyrocarbonate) treated water were combined into a micro-centrifuge tube to a final volume of 12.5 μl. The entire mixture was heated at 70˚C for 10 minutes and chilled on ice for at least 1 minute. The subsequent procedures for conducting reverse transcription were exactly the same as those in the instruction manual (First-strand cDNA synthesis kit, Novagen). The reverse transcription products from total RNA served as a template for PCR. When amplifying target cDNA, components in 50 μl of solution were as follows: 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 20 pmoles of each primer, cDNA template corresponding to the amount synthesized from 50 ng of total RNA and 2 units of DyNAzyme DNA polymerase. The sequence of primers was as follows: caspase-3: GAATACCCTGGACAACA-3' and ACGCC ATGTCATCATCAA; caspase-9: GCCATGGACGAAGCGGAT CGGCGG and GGCCTGGATGAAGAAGAGCTTGGG; β-actin GCTCGTCGTCGACAACGGCTC and CAAACATGATCTGGG TCATCTTCTC (18, 19).



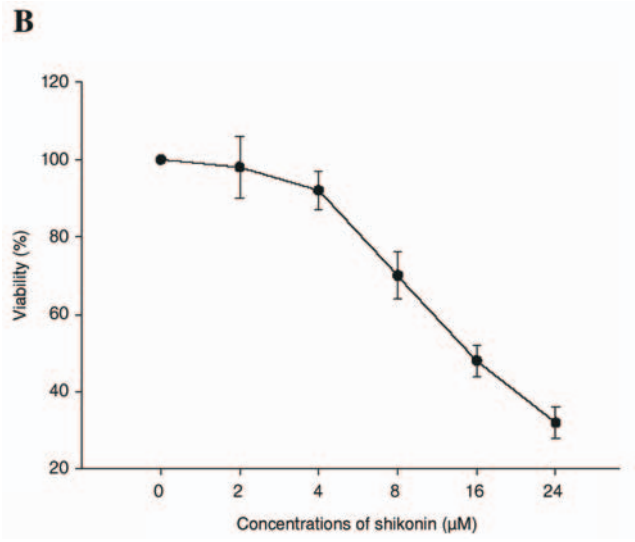


Figure 1. *The percentage of viable T24 cells and morphological changes after 24 hours treatment with shikonin. T24 cells in McCoy's 5a medium + 10% FBS were incubated with different concentrations of shikonin for 24 hours and photographed under a light-phase microscope (panel A). The percentage of viable cells were determined by trypan blue exclusion (panel B) as described in Materials and Methods.*

Western blot analysis. Total protein was prepared and determined. Equal amounts of proteins (30 μg) were separated by SDS-PAGE and transferred onto PVDF membrane (Millipore, MA, USA). The blots were blocked in PBST buffer containing 5% non-fat milk, and then the membrane was incubated overnight with specific primary antibodies: cyclin A, cyclin D, cyclin E, CDK2, CDK4, CDK6 (Santa Cruz Biotechnology, CA, USA) and p21 (Pharmingen, CA, USA). Subsequently, the membrane was washed with PBST buffer and incubated with HRP conjugated secondary antibodies (Santa Cruz Biotechnology). The specific proteins were detected by using Western Blot Chemiluminescence Reagent Plus kits (NEN_ Life Science, MA, USA).

Results

Effects of shikonin on cell viability and morphology. The results from the trypan blue exclusion experiments indicated that <2% of the T24 cells were stained when they were incubated in medium containing 10% FBS. In contrast, in the presence of shikonin (2-24 μM), cell staining increased with concentration, suggesting that shikonin induced death of the T24 cells. Substantial morphological changes were observed in the T24 cells 24 h after the addition of increasing concentrations of shikonin (Figure 1A) characterized by cellular shrinkage. The cell damage appeared after treatment with 8-24 μM of shikonin, while at the same time no pronounced difference from the control regimen was apparent (Figure 1B).

Shikonin induced cell cycle arrest and apoptosis. The data indicated that during a 0-72 h time period, shikonin (16 μM) decreased the percentage of the T24 cells in the S- and G2/M-phases, and increased the percentage in G0/G1-phase. The control cells (without shikonin) showed a typical pattern of DNA content that reflected the normal G0/G1-, S- and G2/M-phases of the cell cycle. The shikonin treated

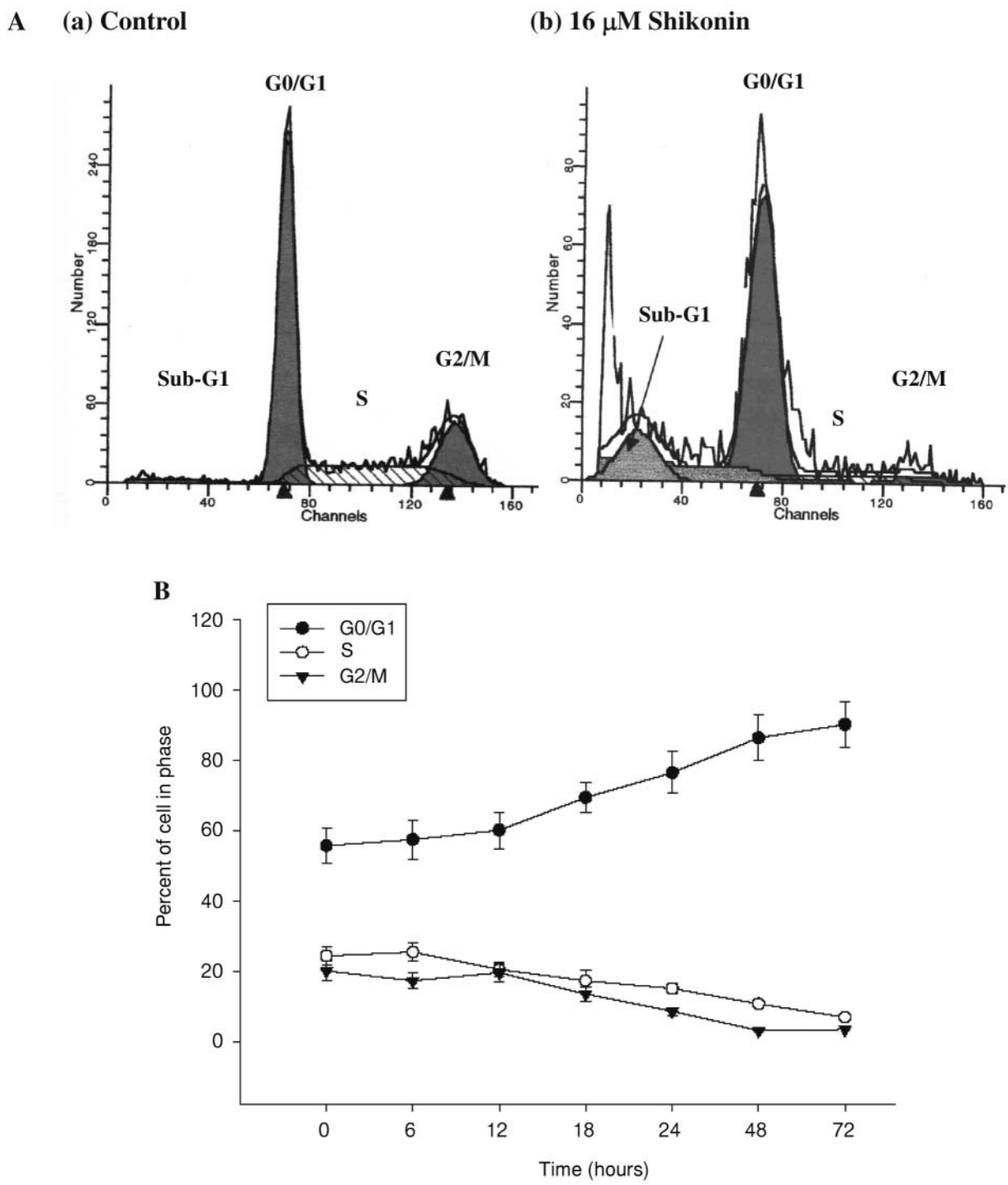


Figure 2. *The effects of shikonin on the cell cycle and sub-G1 group. The T24 cells were incubated with 16 μM shikonin for various time periods and analyzed by flow cytometry (panel A). The percentage of T24 cells in each phase (panel B).*

cells showed a pattern of DNA content that reflected reduced G0/G1-, S- and G2/M-phases together with an increased sub-G0/G1-phase (corresponding to apoptotic cells) as shown in Figure 2A. The percentage of cells in each phase is shown in Figure 2B. The percentage of apoptosis with various concentrations of shikonin and after various times are shown in Figure 3A and 3B. Increasing shikonin concentration and incubation time led to increased

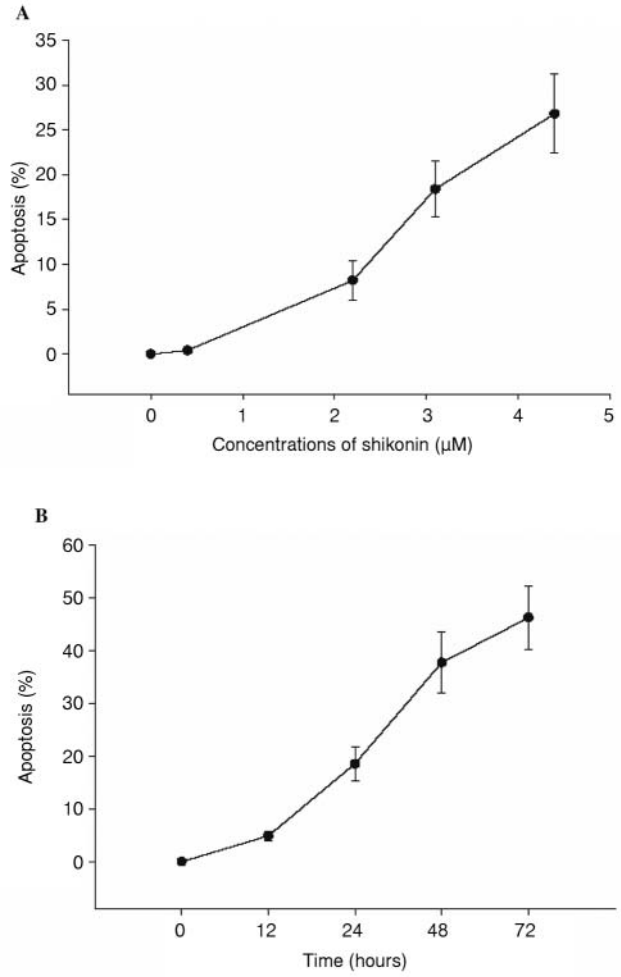


Figure 3. *The effects of shikonin on apoptosis of T24 cells. Shikonin-induced apoptosis (panel A) and the time-course effect of 16 μM shikonin on apoptosis in T24 cells (panel B).*

apoptosis of the T24 cells. A major characteristic of apoptosis is the cleavage of PARP. Apoptosis of the T24 cells was also determined by PARP fluorescence stain. Figure 4 shows *PARP* positive fluorescence at 4, 8, 16 and 24 μM shikonin.

Inhibition of shikonin-induced caspase activity and apoptosis by the caspase inhibitor z-VAD-fmk. The results presented in Figure 5 indicated that shikonin increased caspase-3 activity. Increased incubation time led to increased caspase-3 activity. Pretreatment with Z-VAD-fmk (caspase inhibitor) decreased caspase-3 activity (Figure 6). After cotreatment with shikonin and z-VAD-fmk, inhibition of shikonin-mediated caspase-3 activation was accompanied by the marked attenuation of shikonin-induced apoptotic cell death. The results also indicated that activation of caspase-3 contributed to shikonin-induced apoptosis in the T24 cells.

Effects of shikonin on caspase-3 and -9 mRNA expression examined by PCR and flow cytometry. The changes of caspase-3 and -9 mRNA levels in response to shikonin were studied by PCR (Figure 7A) and by flow cytometry (Figure 7B). The caspase-3 and -9 mRNA levels increased after treatment with 16 μM shikonin (Figure 7A). The protein levels of caspase-9 also increased after treatment with 16 μM shikonin for 24 and 48 h.

Effect of shikonin on the expression of G0/G1-phase involved proteins. The protein expression of p21, CDK2, 4, 6, and cyclin A, cyclin D, and cyclin E revealed by Western blot analysis are presented in Figure 8. After 24-48 h exposure to 16 μM shikonin, an increase of p21 and decrease of cyclin E, CDK2 and CDK4 protein levels in the T24 cell was demonstrated.

Discussion

Based on the results from the present studies, the possible actions of shikonin included inhibition of the viability of T24 cells, induction of apoptosis of the T24 cells *via* promoting caspase-3 and -9 activity, interference with the cell cycle at the G1-phase and with DNA synthesis, by arresting cells at the G0/G1-phase of the cell cycle and thereby preventing them from entering the M-phase. The shikonin-associated increase in the p21 expression and inhibition of cyclin-E, CDK2 and CDK4 proteins may have led to the G0/G1 arrest.

It is well known that cell death has two mechanisms; one is apoptosis (nuclear fragmentation, membrane blebbing and formation of apoptotic bodies) and the other is necrosis (releasing large amounts of intracellular components to the environment). The major difference between them is the active participation of the cells in the process of apoptosis (20, 21). Apoptosis, or programmed cell death, is an essential physiological process that is required for normal development and maintenance of tissue homeostasis (27). When misregulated, apoptosis can contribute to various diseases including cancer, autoimmune and neurodegenerative diseases. Central components of the apoptotic death machinery, which have been conserved throughout evolution, included the Bcl-2, Apaf-1 (apoptotic protease activating factor 1) and caspase family members. Caspases (cysteinyl aspartate specific proteases) are synthesized as dormant proenzymes that, upon proteolytic activation, acquire the ability to cleave key intracellular substrates (27), resulting in the morphological and biochemical changes associated with apoptosis such as DNA degradation into oligonucleosomal fragments (27). The known caspases included caspase -1, -2, -3, -6, -7, -8, -9 and -11 sub-classified into upstream and downstream. Caspase-9 is defined as upstream and caspase-3 as downstream.

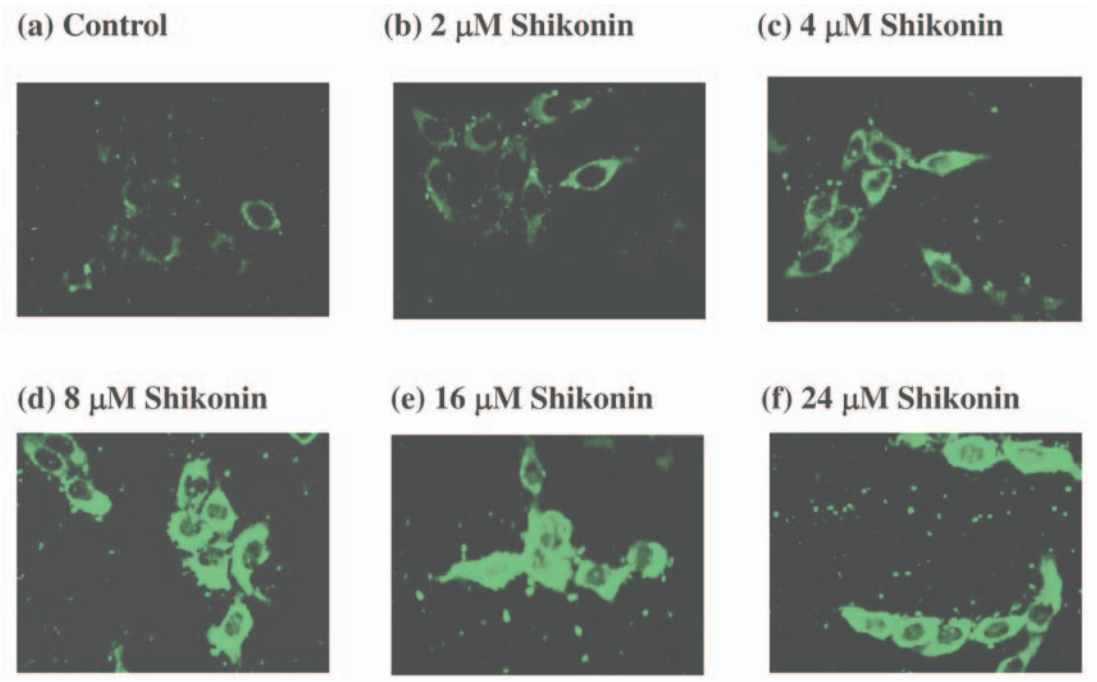


Figure 4. *Shikonin induced poly (ADP-ribose) polymerase (PARP) production. After treatment with or without shikonin T24 cells were fixed, and examined with a PARP monoclonal antibody assay under a fluorescence microscope.*

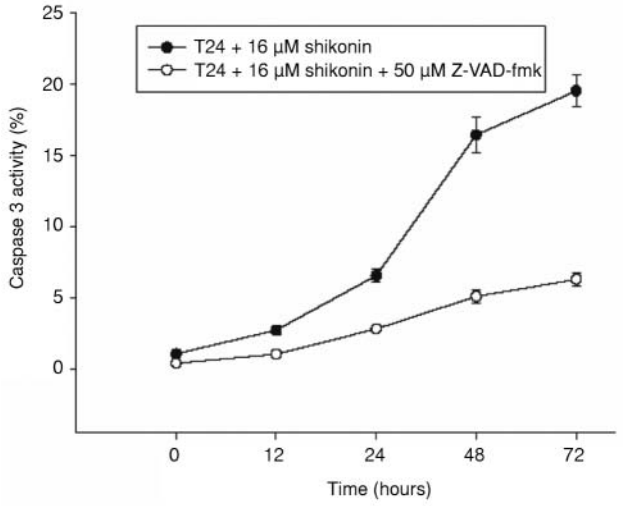


Figure 5. *The effects of shikonin on T24 cell caspase-3 activity. The T24 cells were incubated with 15 μm shikonin and co-treated with or without z-VAD-fmk for various time periods. Then the cells were harvested and were analyzed for caspase-3 activity in T24 cells as described in Materials and Methods.*

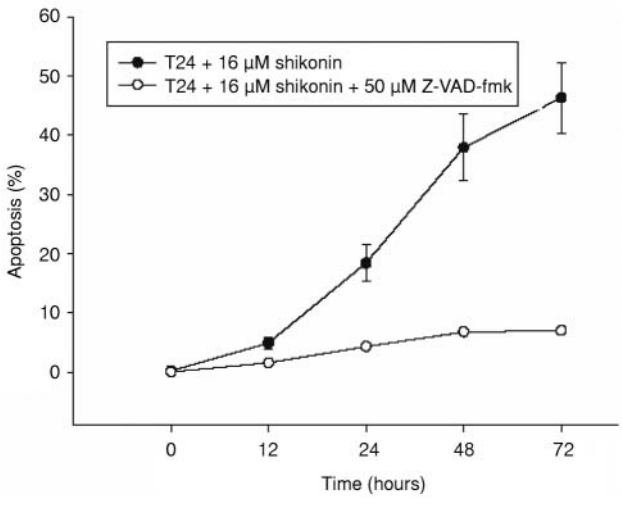


Figure 6. *The effects of z-VAD-fmk on shikonin induced apoptosis. The T24 cells were incubated with 16 μm shikonin after 3 h pretreatment with or without z-VAD-fmk, for various time periods, and then analyzed for apoptosis.*

Many investigators have already pointed out that internucleosomal DNA fragmentation is not unique to apoptotic cell death since it may also be present in necrotic cell death. Therefore, it has been suggested that internucleosomal DNA fragmentation may not be enough as an indicator of apoptotic cell death (22, 23). From p10 and by this measurement the results of the present study also demonstrated that shikonin did induce apoptosis in the

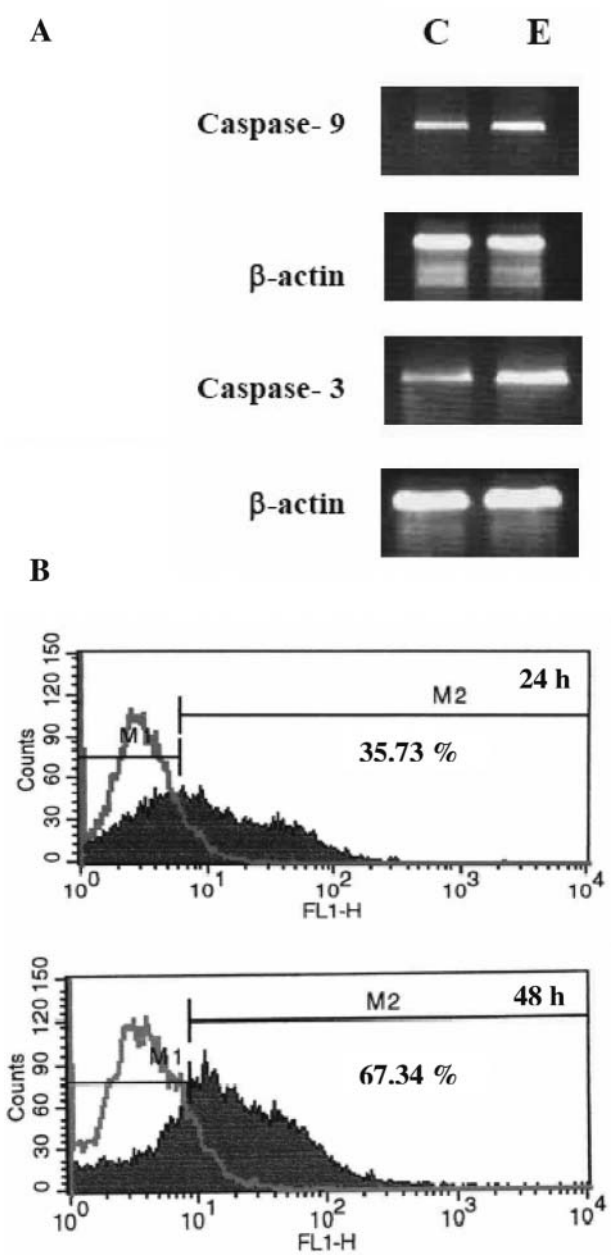


Figure 7. *(A) The effect of shikonin on the expression of caspase-3 and -9 mRNA in T24 cells. The caspase-3 and -9 bands were strengthened in comparison with β-actin after treatment with shikonin. (B) The caspase-9 activity in T24 cells. Caspase-9 activity in T24 cells was also estimated by flow cytometry.*

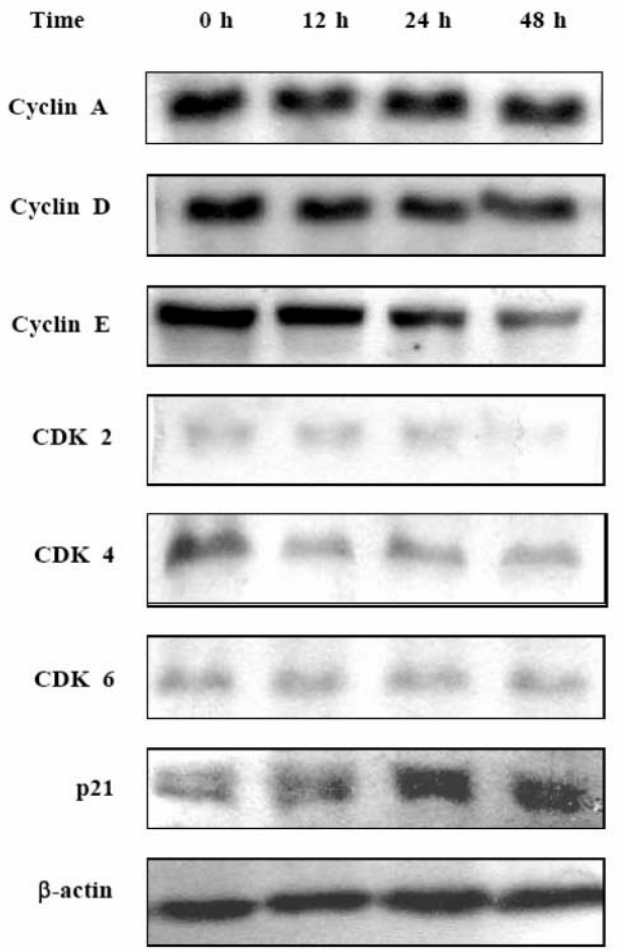


Figure 8. *Effects of shikonin on the expression of proteins involved in G0/G1-phase and on cell cycle progression. Western blot analysis of cyclin A, cyclin D, cyclin E, CDK2, CDK4, CDK6, p21 and β-actin protein expression levels. Total cellular extracts were subjected to SDS-PAGE and immunoblotted with antibodies specific to human cyclin A, cyclin D, cyclin E, CDK2, CDK4, CDK6, p21 and β-actin.*

T24 cells (Figure 4). The shikonin-induced cell death was mediated by caspase-3 and caspase-9 activation and was therefore an expression of apoptosis as revealed by flow cytometric assay (Figure 2A) where the sub-G1 group appeared in the cell cycle. Importantly the internucleosomal DNA fragmentation (apoptosis) was completely inhibited by the pretreatment with caspase inhibitor (z-VAD-fmk) (Figure 3A and 6). It has been demonstrated that shikonin-induced cell apoptosis is caspase-3 dependent and caspase-3 activation is a downstream caspase program (24). These results are in agreement with the report of Yoon *et al.* (11), which showed that shikonin induced apoptosis in HL-60 a human premyelocytic leukemia cell line and all the features of apoptosis could be blocked by z-VAD-fmk. Other investigators have also reported that the ratio between pro- and anti-apoptotic proteins determines, in part, the susceptibility of cells to a death signal (24-26). The activation of caspase-3 induced caspase-activatable DNase (CAD) and led to chromosomal DNA breaking and finally induced apoptosis.

It has been reported that some cellular processes involved in the cell cycle play an important role in the signal transmission pathway of apoptosis and in particular mitochondrial cytochrome *c* release is an important control point in caspase activation and apoptosis (27). It has been suggested that susceptibility to the apoptosis-inducing effects of chemotherapeutic drugs may depend on the intrinsic ability of tumor cells to respond by apoptosis (20). Yano *et al.* also pointed out that defects in the process of apoptosis may be closely associated with carcinogenesis and that many cancer cells have defective machinery for self-destruction. Advance of the cell cycle in multicellular organisms has been linked to a family of protein kinases such as cyclin-dependent kinases and cyclins that are involved in the regulation of different checkpoints (28-30). Many reports have shown that cyclin-dependent kinase 2 (CDK2) and cyclin-E are involved in the transition from G1- into S-phase, and our data did show that shikonin increased the p21 expression and inhibited cyclin-E, CDK2 and CDK4 protein expression which may be the factor that led to G0/G1 arrest in the T24 cells. Furthermore, shikonin was reported to show anticancer activity decades ago (31) and it has been on clinical trial for later stage lung cancer (32).

Conclusion

In these studies, shikonin was shown to induce G0/G1 arrest and apoptosis of human bladder cancer cells (T24) and it is suggested that shikonin could be used effectively for the treatment of human cancer in the future.

Ackowledgements

This study was supported by grant DMR-092-067 from the Research Section of the China Medical Unversity Hospital, Taichung city, Taiwan, R.O.C.

References

1 Bhalla K, Ibrado AM, Tourkina E, Tang C, Mahoney ME and Huang Y: Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. Leukemia *7*: 563-568, 1993.

2 Kaufman SH: Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res *49*: 5870-5878, 1989.

3 Walker PR, Smith C, Youdale T, Leblanc J, Whitfield JF and Sikorska M: Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. Cancer Res *51*: 1078-1085, 1991.

4 Ling YH, Priebe W and Perze SR: Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. Cancer Res *53*: 1845-1852, 1993.

5 Stevnsner T and Bohr VA: Studies on the role of topoisomerase in general, gene and strand-specific DNA repair. Carcinogenesis *14*: 1841-1850, 1993.

6 Kerr JFR, Wyllie AH and Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*: 239-257, 1972.

7 Seto Y, Motoyoshi S, Nakamura H, Imuta J, Ishitoku T and Isayama S: Effect of shukonin and its derivatives, pentaacetylated and shikonin (MDS-004) on granuloma formation and delayed-type allergy in experimental animals. Yakugaku Zasshi *112*: 259-271, 1992 (in Japanese).

8 Wang, WJ, Bai JY, Liu DP, Xue LM and Zhu XY: The antiinflammatory activity of shikonin and its inhibitory effect on leukotriene B4 biosynthesis. Yao Xue Xue Bao *29*: 161-165, 1994.

9 Ahn BZ, Baik KU, Kweon GR, Lim K and Hwang BD: Acylshikonin analogues: synthesis and inhibition of DNA topoisomerase-I. J Med Chem *17*: 1044-1047, 1995.

10 Hisa T, Kimura Y, Takada K, Suzuki F and Takigawa M: Shikonin, an ingredient of *Lithospermum erythrorhizon*, inhibits angiogenesis *in vivo* and *in vitro*. Nippon Yakurigaku Zasshi *73*: 193-203, 1998.

11 Yoon Y, Kim YO, Lim NY, Jeon WK and Sung HJ: Shikonin, an ingredient of *Lithospermum erythrorhizon* induced apoptosis in HL60 human premyelocytic leukemia cell line. Planta Medica *65*: 532-535, 1999.

12 Subbaramaiah K, Bulic P, Lin Y, Dannenberg AJ and Pasco DS: Development and use of a gene promoter-based screen to identify novel inhibitors of cyclooxygenase-2 transcription. J Biomol Screen 6: 101-110, 2001.

13 Gaddipati JP, Mani H, Shefali RK, Mathad VT, Bhaduri AP and Maheshwari PK: Inhibition of growth and regulation of IGFs and VEGF in human prostate cancer cell lines by shikonin analogue 93/637 (SA). Anticancer Res *20*: 2547-2552, 2000.

14 Pettit GR, Hoard MS, Doubek DL, Schmidt JM, Pettit RK, Tackett LP and Chapuis JC: Antineoplastic agents 338: the cancer cell growth inhibitory constituents of Terminnalia arjuna. J Ethnopharmaco *53*: 57-63, 1996.

15 Ormerod MG: Flow Cytometry: A Practical Approach. Oxford University Press, New York, pp. 69-81, 1990.

16 Chung JG: Effects of the butylated hydroxyanisole and butylated hydroxytoluene on the acetylation of 2-aminofluorene and DNA-2-aminofluorene adducts in the rat. Toxicological Science *51*: 202-210, 1999.

17 Hsia TC, Chung JG, Lu HF, Ho HC, Yang CC, Lu KH and Hung CF: The effect of paclitaxel on 2-aminofluorene-DNA adducts formation and arylamine N-acetyltransferase activity and gene expression in human lung tumor cells (A549). Food and Chem Toxicol *40*: 697-703, 2002.

18 Uehara T, Kikuchi Y and Nomura Y: Caspase activation accompanying cytochrome c release from mitochondria is possibly involved in nitric oxide-induced neuronal apoptosis in SH-SY5Y cells. J Neurochem *72*: 196-205, 1999.

19 Kuida A, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MSS, Rakis P and Flavell RA: Reduced apoptosis and cytochrome-c-mediated caspase activation in mice lacking caspase 9. Cell *94*: 325-337, 1998.

20 Yano H, Mizoguchi A, Fukuda K, Haramaki M, Ogasawara S, Momosaki S and Kojiro M: The herbal medicine sho-saiko-to inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G0/G1 phase. Cancer Res *54*: 448-454, 1994.

21 McGill G, Shimamura A, Bates RC, Savage RE and Fisher L: Loss of matrix adhesion triggers rapid transformation-selective apoptosis in fibroblasts. J Cell Biol *138*: 901-911, 1997.
22 Cohen GM, Sun XM, Snowden RT, Dinsdale D and Skilleter DN: Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem J *286*: 331-334, 1992.
23 Schulze-Osthoff K, Wakzak H, Droge W and Krammer PH: Cell nucleus and DNA fragmentation are not required for apoptosis. J Cell Biol *127*: 15-20, 1994.
24 Gross A, McDonnell JM and Korsmeyer SJ: BCL-2 family members and the mitochondria in apoptosis. Genes Dev *13*: 1899-1911, 1999.
25 Vander Heiden MG and Thompson CB: Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis. Nat Cell Biol *1*: E209-216, 1999.
26 Zhang H, Huang Q, Ke N, Matsuyama S, Hammock B, Godzik A and Reed JC: Drosophila pro-apoptotic Bcl-2/Bax homologue reveals evolutionary conservation of cell death mechanism. J Biol Chem *275*: 27303-27306, 2000.
27 Solange D and Martinou JC: Mitochondria as the central control point of apoptosis. Trends. Cell Biol *10*: 369-377, 2000.
28 Lee YS, Kim WH, Yu ES, Kim MR, Lee MJ and Jang JJ: Time course of cell cycle-related protein expression in diethylnitrosamine-initiated rat liver. J Hepatol *29*: 464-469, 1998.
29 Choi KS, Eom YW, Kang Y, Ha MJ, Rhee H, Yoon JW and Jim SJ: Cdc2 and Cdk2 kinase activated by transforming growth factor-beta 1 trigger apoptosis though the phosphorylation of retinoblastoma protein in FaO hepatoma cells. J Biol Chem *274*: 31775-31783, 1999.
30 Barnes EA, Kong M, Ollendorff V and Donoghue DJ: Patched 1 interacts with cyclin B1 to regulate cell cycle p rogression. EMBO J *20*: 2214-2223, 2001.
31 Sankawa U, Ebizuka Y, Miyazaki T, Isomura Y and Otsuka H: Antitumor activity of shikonin and its derivatives. Chem. Pharm Bull (Tokyo) *25*: 2392-2395, 1977.
32 Guo XP, Zhang XY and Zhang SD: Clinical trial on the effects of shikonin mixture on later stage lung cancer. Zhong Xi Yi Jie He Za Zhi (in Chinese) *11*: 598-599, 1991.

Received May 29, 2007
Revised September 5, 2007
Accepted September 11, 2007