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Induction of Apoptosis by Shikonin through Coordinative Modulation of the Bcl-2 Family, p27, and p53, Release of Cytochrome *c*, and Sequential Activation of Caspases in Human Colorectal Carcinoma Cells

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Shikonin is a main constituent of the roots of *Lithospermum erythrorhizon* that has antimutagenic activity. However, its other biological activities are not well-known. Shikonin displayed a strong inhibitory effect against human colorectal carcinoma COLO 205 cells and human leukemia HL-60 cells, with estimated IC_{50} values of 3.12 and 5.5 μM, respectively, but were less effective against human colorectal carcinoma HT-29 cells, with an estimated IC_{50} value of 14.8 μM. Induce apoptosis was confirmed in COLO 205 cells by DNA fragmentation and the appearance of a sub-G1 DNA peak, which were preceded by loss of mitochondrial membrane potential, reactive oxygen species (ROS) generation, cytochrome *c* release, and subsequent induction of pro-caspase-9 and -3 processing. Cleavages of poly(ADP-ribose) polymerase (PARP) and DNA fragmentation factor (DFF-45) were accompanied by activation of caspase-9 and -3 triggered by shikonin in COLO 205 cells. Here, we found that shikonin-induced apoptotic cell death was accompanied by upregulation of p27, p53, and Bad and down-regulation of Bcl-2 and Bcl-X_L, while shikonin had little effect on the levels of Bax protein. Taken together, we suggested that shikonin-induced apoptosis is triggered by the release of cytochrome *c* into cytosol, procaspase-9 processing, activation of caspase-3, degradation of PARP, and DNA fragmentation caused by the caspase-activated deoxyribonuclease through the digestion of DFF-45. The induction of apoptosis by shikonin may provide a pivotal mechanism for its cancer chemopreventive action.

KEYWORDS: Shikonin; apoptosis; cytochrome *c*; caspase-9; caspase-3; poly-(ADP-ribose) polymerase; DNA fragmentation factor; caspase-activated deoxyribonuclease; Bcl-2; Bcl-X_L

INTRODUCTION

Epidemiological studies have provided convincing evidence that dietary factors can modify the processes of carcinogenesis, including initiation, promotion, and progression of several types of human cancer (*1*). The occurrence of gastrointestinal (GI) cancers has increased strikingly during the past decade. For instance, colorectal cancer is the second leading cause of cancer mortality in Western societies (*2*) and one of the world's most common malignancies (*3*, *4*). Therefore, fighting against GI cancer is an important global issue. During the past decade, a large number of natural products and dietary components have been evaluated as potential chemopreventive agents (*5*). Medicinal plants have been used as traditional remedies for hundreds of years. Shikonin (**Figure 1**), a pigment originally isolated from the root of the Chinese plant *Lithospermum erythrorhixon*, was reported to exhibit antitumor (*6*, *7*) and antiinflammatory activities (*8*). In addition, Shikonin has been proven to possess strong wound healing and antibacterial activities (*9*), and is associated with apoptosis (*10*, *11*) and

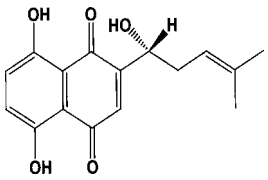

Figure 1. Chemical structure of shikonin.

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modulated cell proliferation (*12*). Although a broad range of biological and pharmacological activities of shikonin have been reported, the mechanism(s) by which shikonin induces apoptosis is not yet known.

Apoptosis, a morphologically distinct form of programmed cell death, is an evolutionary highly conserved phenomenon that plays an important role in the regulation of cellular activities in eukaryotes (*13*). Apoptosis is also a gene-directed form of cell death with well-characterized morphological and biochemical features (*14*). Initiation of apoptosis appears to be a common mechanism of many cytotoxic agents used in chemotherapy. A family of cytosolic proteases, the caspases, stored in most cells as zymogens, plays an essential role in the execution of apoptosis. Proteolytic cleavage activates the initiating zymogen, which in turn triggers sequential proteolytic activation of each successive procaspase in the apoptosis cascade (*15*−*17*). There are two main pathways involving apoptotic cell death: One is the interaction of the cell surface receptor, such as Fas, TNFR, DR3, DR4, and DR5 (*18*), with their ligands, and the second pathway involved the participation of mitochondria. The mitochondrial pathway was regulated by the Bcl-2 family proteins, including anti-apoptoic proteins such as Bcl-2 and Bcl-X_L and pro-apoptotic proteins such as Bax, Bad, Bak, and Bid (*19*). Specifically, damage to mitochondria results in the release of cytochrome *c*, which together with Apaf-1 (apoptotic protease activating factor-1) and dATP lead to the recruitment and activation of pro-caspase-9 (*20*, *21*). Apaf-1 was the first identified mammalian homologue of Ced-4 that couples cytochrome *c* to activation of caspase-9 (*20*). Most evidence suggests that each cell type has a set point for the ratio of Bcl-2 family dimers, such as levels of Bcl-2 to Bax, which gauges the sensitivity of a cell toward survival or apoptosis. In many cells, survival or death depends on the altered expression level of death inhibitor to death promoter, respectively. Furthermore, it appears that a range of molecular affinities exist which control the interactions between family members, such as Bcl-2 (or Bcl-X_L) for Bax to promote cell survival or Bax homodimer formation to promote cell death (*22*, *23*). Current evidence suggests that Bcl-2 acts upstream of caspse-3 activation, at the level of cytochrome *c* release, to prevent apoptosis (*24*). It has been shown that the Bcl-2 and Bcl-X_L of mammals can be converted into potent proapoptotic molecules when they are cleaved by caspases and result in accelerating cell apoptosis (*24*, *25*).

In this study, we first examined the antiproliferative effects of shikonin on human colorectal carcinoma cells. Our results clearly demonstrate that shikonin can induce apoptosis in a dose-dependent manner in COLO 205 cells. We further evaluated the molecular mechanisms of apoptotic effects induced by shikonin. To elucidate the anticancer mechanism of shikonin, we investigated the change of the Bcl-2 protein family and caspases in shikonin-induced apoptosis in human COLO 205 cancer cells.

MATERIALS AND METHODS

Cell Culture and Chemicals. The cell lines COLO 205 (CCL-222; American Type Culture Collection) were developed from a poorly differentiated human colon adenocarcinoma. HT-29 (HTB-38; American Type Culture Collection [ATCC], Rockville, MD) was isolated from moderately well-differentiated grade II human colon adenocarcinoma. HL-60 was developed from human myeloid leukemia cells. Cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL, Grand Island, NY), 100 units/mL of penicillin, 100 μg/mL of streptomycin), 2 mM L-glutamine (GIBCO BRL, Grand Island, NY), and were kept at 37 °C in a humidified 5% CO_2 incubator. Shikonin was purchased form Calbiochem (La Jolla, CA), and its purity was >97%.

Cell Survival Assay. Cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, COLO 205 cells were plated at a density of 1×10^5 cells/mL into 24-well plates. After overnight growth, cells were pretreated with a series of concentrations of shikonin for 12 h. The final concentrations of dimethyl sulfoxide in the culture medium were <0.1%. At the end of treatment, 30 μL of MTT was added, and cells were incubated for a further 4 h. Cell viability was obtained by scanning with an ELISA reader with a 570 nm filter.

DNA Extraction and Electrophoresis Analysis. COLO 205 human cancer cells were harvested, washed with PBS, and then lysed with digestion buffer containing 0.5% sarkosyl, 0.5 mg/mL proteinase K, 50 mM tris(hydroxymethyl)aminomethane (pH 8.0), and 10 mM EDTA at 56 °C overnight and were treated with RNase A (0.5 μg/mL) for 3 h at 56 °C. The DNA was extracted by phenol/chloroform/isoamyl (25/24/1) before loading and was analyzed by 2% agarose gel electrophoresis. The agarose gels were run at 50 V for 120 min in Tris-borate/EDTA electrophoresis buffer (TBE). Approximately 20 μg DNA was loaded in each well and visualized under UV light and photographed (*26*).

Flow Cytometry. COLO 205 cells (2×10^5) were cultured in 60-mm Petri dishes and incubated for 12 h. The cells were then harvested, washed with PBS, resuspended in 200 μL of PBS, and fixed in 800 μL of iced 100% ethanol at −20 °C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at 37 °C for 30 min. Next, 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide−DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA).

DHE Staining. Cells were incubated with 20 μM DHE in tissue culture medium for 45 min at 37 °C and then washed, resuspended as 1×10^6/mL in PBS, and submitted to flow analysis.

Analysis of Mitochondrial Transmembrane Potential. The change of the mitochondrial transmembrane potential was monitored by flow cytometry. Briefly, COLO 205 cells were exposed to shikonin (10 μM) for 1 h, and the mitochondrial transmembrane potential was measured directly using 40 nM 3,3′-dihexyloxacarbocyanine [DiOC6(3)] (Molecular Probes, Eugene, OR). Fluorescence was measured after staining of the cells for 15 min at 37 °C. Histograms were analyzed using Cell Quest software and were compared with histograms of control untreated cells.

Western Blotting. The nuclear and cytosolic proteins were isolated from COLO 205 cells after treatment with 10 μM or 20 μM for 0, 2, 4, 6, 8, 10, and 12 h. The total proteins were extracted via addition of 200 μL of gold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10 000*g* for 30 min at 4 °C. The cytosolic fraction (supernatant) proteins were measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and were subjected to 12% SDS-polyacrylamide minigels at a constant current of 20 mA. Electrophoresis was ordinarily carried out on SDS-polyacrylamide gels. Following electrophoresis, proteins on the gel were electrotransferred onto an immobile membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris-HCl, and then immunoblotted with primary antibodies including anti-Bcl-2, anti-Bcl-x_L, anti-Bad, anti-Bax, anti-α-tubulin (Santa Cruz Biotech.), anti-PARP (UBI, Inc., Lake Placid, NY), anti-p21 (Transduction Laboratory, Lexington, KY), and anti-DFF45/inhibitor of caspase activated DNase (ICAD) antibody (MBL, Naka-Ku, Nagoya, Japan) at room temperature for 1 h. Detection was achieved by measuring the chemiluminescence of blotting agent (ECL, Amersham Corp., Arlington Heights, IL), after exposure of the filters to Kodak X-Omat films. The mitochondria and cytosolic fractions

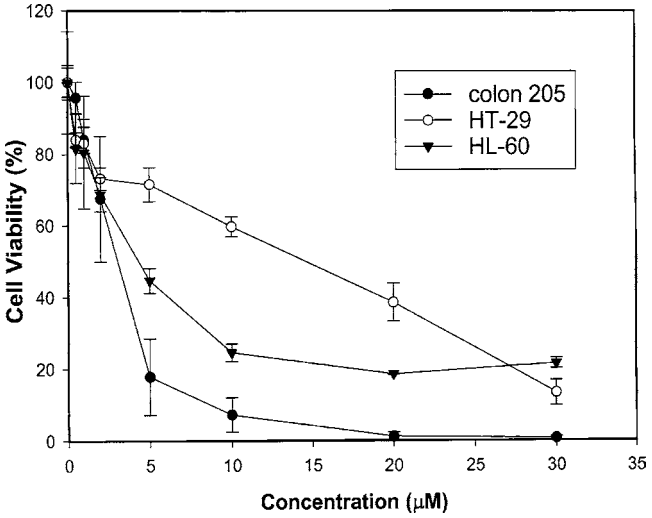

Figure 2. Effects of shikonin on the cell viability. HL-60, COLO 205, and HT-29 cells were treated with shikonin at different concentrations for 12 h. Cells were treated with 0.1% DMSO as vehicle control. Viability of the cells was then determined by MTT assay, as described in the Materials and Methods. Data were represented as means ± SE for three determinations.

isolated from cells were used for immunoblot analysis of cytochrome *c* as described. The cytochrome *c* protein was detected by using anti-cytochrome *c* antibody (Research Diagnostic Inc., Flanders, NJ).

Activity of Caspase. Cells were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM MgCl, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at 12 000*g* for 20 min at 4 °C. Caspase activity in the supernatant was determined by a fluorogenic assay (Promeaga's CaspACE Assay System Corp., Madison, WI). Briefly, 50 μg of total protein, as determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany), was incubated with 50 μM substrate Ac-Try-Val-Ala-Asp-AMC (Ac-YVAD) (caspase-1 specific substrate), Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) (caspase-3 specific substrate), Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) (caspase-8 specific substrate), or Ac-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) (caspase-9 specific substrate) at 30 °C for 1 h. The release of methylcoumaryl-7amine (AMC) was measured by excitation at 360 and emission at 460 nm using a fluorescence spectrophotometer (HITACHI, F2000).

RESULTS

Treatment with Shikonin Causes Dose-Dependent Reduction in Cell Viability. We first investigated the effect of shikonin on cell viability; human colorectal carcinoma cell lines COLO 205 or HT 29 and human leukemia cell line HL-60 were treated with different concentrations of shikonin. After 12 h of treatment, the number of live cells was determined by means of the MTT test. As shown in **Figure 2**, shikonin appeared to be more potent inhibitor of COLO 205 cell viability with an IC_{50} of 4.3 μM, and inhibition of the cell viability was found to be dose-dependent. However, HL-60 and HT-29 cells were more resistant to shikonin; the IC_{50} values were 5.5 and 14.8 μM, respectively. The morphological changes in COLO 205 cells treated with 10 μM shikonin in this study are illustrated in **Figure 3**. The cells shrunk during the time period from 4 to 12 h, further confirming the occurrence of cell death after shikonin treatment.

Shikonin Induced DNA Fragmentation of COLO 205 Cells. Physiological cell death is characterized by apoptotic morphology, including chromatin condensation, membrance blebbing, internucleosome degradation of DNA, and apoptotic

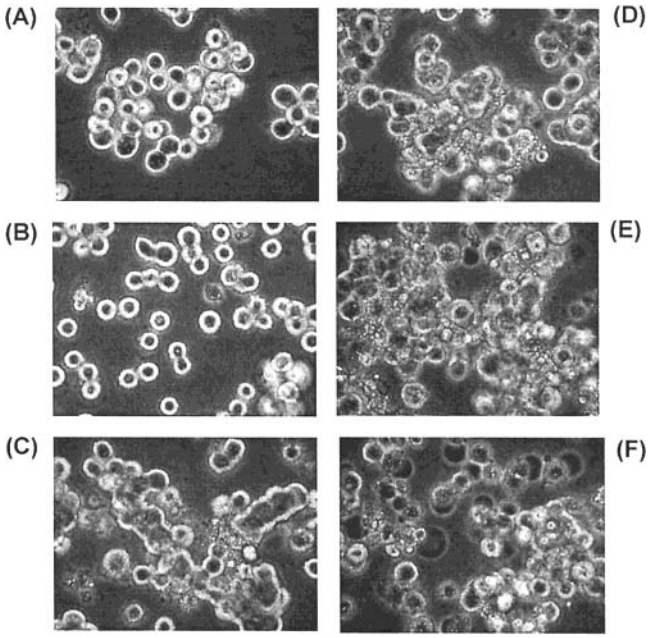

Figure 3. Morphological changes of COLO 205 cells induced by 10 μM shikonin. The morphological changes of COLO 205 cells were detected by light microscopic observation. (**A**) 0 h; (**B**) 2 h; (**C**) 4 h; (**D**) 6 h; (**E**) 8 h; (**F**) 12 h (100× original magnification).

body formation. To determine whether the inhibition of cell growth by shikonin resulted from the induction of apoptosis, DNA fragmentation, a hallmark of apoptosis, was demonstrated by incubating COLO 205 cells with different concentrations of shikonin for 12 h (**Figure 4**); the genomic DNA from cells was subjected to agarose gel electrophoresis. A clear DNA fragmentation ladder was found in ethidium-stained gels, at 5 μM shikonin treatment, and this DNA fragmentation response was dose-dependent. When cells were treated with 10 μM shikonin, DNA ladders were just visible 8 h after treatment (**Figure 4B**).

A sub-G1 (sub-2N) DNA peak, which has been suggested to be the apoptotic DNA (*27*), was detected in cells that were treated with shikonin (10 μM), washed, and stained with propidium iodide. As shown in **Figure 5**, the percentages of apoptotic COLO 205 cells (right column) were gradually increased after 0, 6, 8, 10, and 12 h of incubation with shikonin as compared to untreated cells (0.05% DMSO, left column). The percentages of apoptotic COLO 205 cells were 7.42%, 13.54%, 22.02%, 31.15%, and 57.93%, respectively, after incubation with shikonin (10 μM). The percentages of apoptotic COLO 205 cells were 5.04%, 9.45%, 6.28%, 6.02%, and 7.74% without shikonin (control), respectively. The peak of apoptosis did not appear until after 8 h of incubation with shikonin, and this timing is consistent with the appearance of the DNA ladder. Taken together, these results indicated that shikonin induced typical apoptosis in COLO 205 cells.

Disruption of Mitochondrial Membrane Potential and ROS Generation in Shikonin-Induced Apoptosis. The alteration of mitochondrial function is linked to apoptosis, and a decreasing mitochondrial transmembrane potential ($\Delta\Psi$m) is associated with mitochondrial dysfunction (*28*). Thus, we next evaluated the change of $\Delta\Psi$m in shikonin-induced apoptosis. We measured $\Delta\Psi$m using the fluorescent probe [DiOC6(3)] fluorescence and monitored it using flow cytometry. As shown in **Figure 6A**, COLO 205 cells were treated with 10 μM of shikonin for 1 h and exposed to [DiOC6(3)] and displayed that a reduction in $\Delta\Psi$m was detected at 30 min and clearly changed in [DiOC6(3)] fluorescence (the means of fluorescent intensity

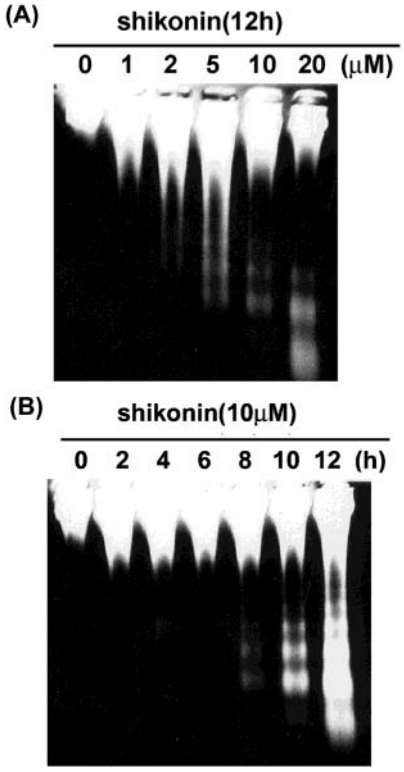

Figure 4. Effect of shikonin on DNA fragmentation of COLO 205 cells. Induction of DNA fragmentation in COLO 205 cells by shikonin. (**A**) Treated with the shikonin of different concentrations for 12 h. (**B**) Time-dependent increasing dose of fragmentation ladders induced by 10 μM shikonin. Agarose gel analysis of DNA fragmentation was performed.

were shifted from 30.28 to 120.08 as compared to control cells, respectively).

Furthermore, we studied the loss of mitochondrial transmembrane potential resulting in the generation of ROS by assessing ROS generation using the fluorescent probe DHE, which is oxidized in the presence of superoxide (O_2^-) to fluorescent products (*29*), and by monitoring via flow cytometry. COLO 205 cells were treated with 10 μM shikonin for 1 h, followed by addition of 20 μM HE into the medium for a further 0.5 h (**Figure 6B**). An increase of intracellular peroxide levels by 10 μM shikonin was detected for 1 h and clearly increases the mean of DHE fluorescence intensity from 121.45 (MC, control) to 375.91 (MS, shikonin). These data indicated that the increment of ROS might play a role as an early mediator in shikonin-induced apoptosis. These findings point to an effect of shikonin on mitochondrial function and accumulation of ROS. These features are cues for the induction of apoptosis.

Shikonin Induction of Cytochrome *c* Release and Caspase-9 Activation. Disruption of mitochondrial membrane potential ($\Delta\Psi$m) results from opening of permeability transition pores, causing a local disruption of the outer mitochondrial membrane, and leads to the release of soluble intermembrane proteins, including cytochrome *c*, and the cytochrome *c* release contributes to the activation of caspase-9 and subsequently causes apoptosis (*19*). To determine whether the reduction of $\Delta\Psi$m induced by shikonin could cause the release of cytochrome *c*, we next evaluated the effect of shikonin on the mitochondrial cytochrome *c* release into the cytosol. Treatment of COLO 205 cells was performed with different concentrations of shikonin for 12 h. As shown in **Figure 7A**, the release of cytochrome *c* into cytosol by shikonin was highly significant and dose-dependent. However, the level of cytochrome *c* in cytosol declined in 10 μM shikonin-treated cells for 12 h, possibly as a result of loss of cytoplasm by acute necrotic cell lysis. Further treatment of COLO 205 cells with 10 μM shikonin caused a clear increase of cytochrome *c* into cytosol at 2 h. Furthermore, we demonstrated whether the cytochrome *c* release resulting from shikonin treatment would subsequently result in the cleavage and activation of caspase-9; the cleavage of pro-caspase-9 was detected at different concentrations of shikonin for 12 h (**Figure 7C**). A dose-dependent proteolytic cleavage of pro-caspase-9, with an increase of the cleaved fragment, was associated with the activity of caspase-9. Consistent with the timing of the cytochrome *c* release, the cleavage of pro-caspase-9 sequentially occurred in COLO 205 cells exposed in a time-course-dependent manner (**Figure 7D**). These observations suggested that an apoptosis-inducing mechanism via mitochondria triggered by shikonin operates in COLO 205 cell lines.

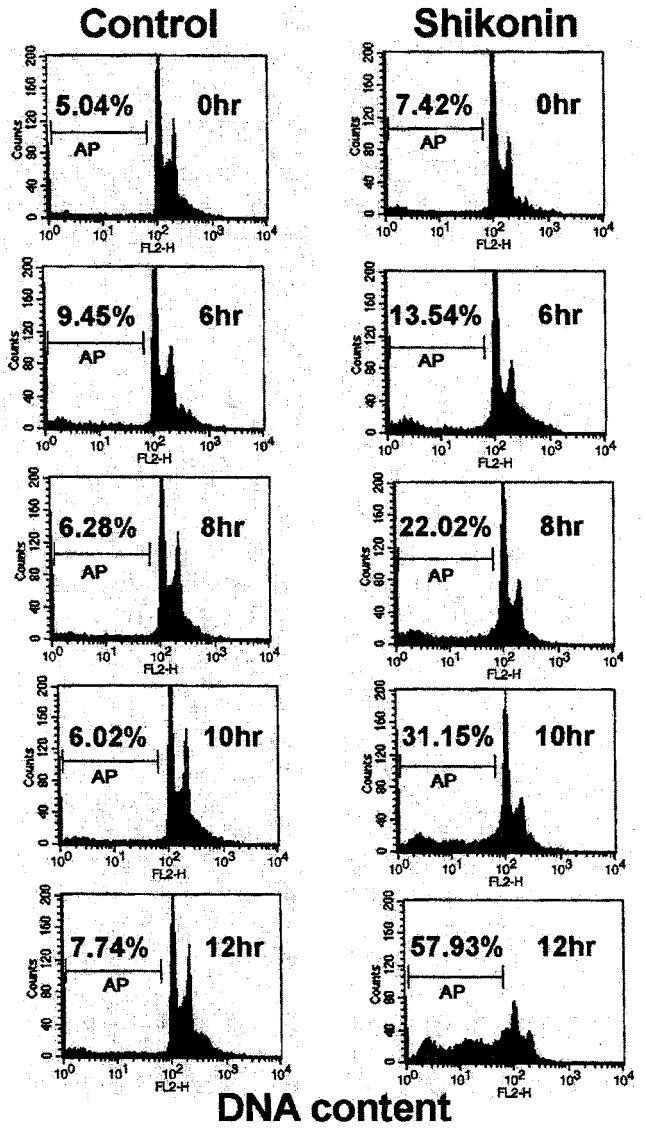

Figure 5. Determination of sub-G1 cells in control and shikonin-treated COLO 205 cells by flow cytometry. COLO 205 cells were treated with 10 μM shikonin or with 0.05% DMSO as vehicle control for 0, 6, 8, 10, and 12 h, and the ratio of sub-G1 cells in shikonin-treated COLO 205 cells was analyzed by flow cytometry. The method of flow cytometry used is described under Materials and Methods. AP (apoptotic peak) represents apoptotic cells with a lower DNA content. The data presented are representative of three independent experiments.

Shikonin Stimulated Caspase-3 Activity in a Dose- and Time-Dependent Manner. Because activation of caspase-9 is necessary for the processing and activation of caspase cascades,

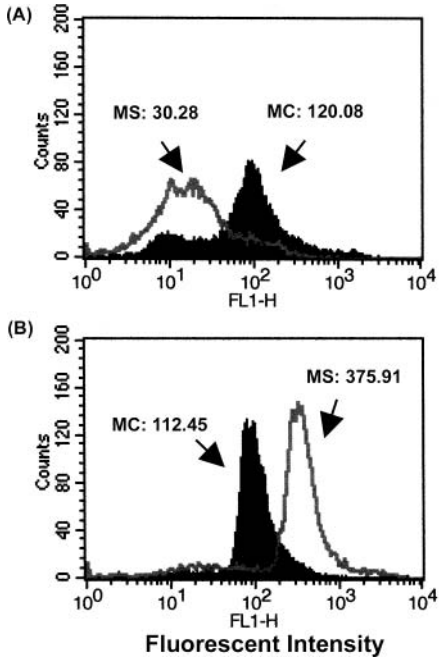

Figure 6. Induction of mitochondrial dysfunction and reactive oxygen species (ROS) generation in COLO 205 by 10 μM shikonin. (**A**) Cells were treated with 10 μM shikonin for 1 h and incubation with 40 nM 3,3′-dihexyloxacarbocyanine and analyzed by flow cytometry. (**B**) COLO 205 cells were treated with shikonin (10 μM) for 1 h and with 20 μM DHE; the fluorescence in the cells was immediately assayed using flow cytometry. MC, control; MS, shikonin. The number represented the mean of relative fluorescent intensity. The data presented are representative of three independent experiments.

we next evaluated whether the processing and activation of caspase-3, which was considered to play a central role in many types of stimuli-induced apoptosis (*19*, *30*, *31*), was associated with the activation of caspase-9 in shikonin-induced apoptosis. We used a fluorogenic peptide substrate, Ac-DEVD-AMC, specific for caspase-3. As shown in **Figure 8A**, caspase-3 was dose-dependently activated by shikonin, and the activity of caspase-3 could be seen at 8 h after the addition of shikonin.

Treatment with Shikonin Caused Degradation of PARP, an Endogenous Substrate of Caspase-3, and Cleavage of DFF-45, an Inhibitor of Endonuclease. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is poly-(ADP-ribose) polymerase (PARP). The cleavage of PARP is the hallmark of apoptosis. PARP (116 kDa) is cleaved to produce an 85-KDa fragmentation during apoptosis (*32*). As was already described, ICAD is a mouse homologue of human DFF-45, caspase-3 cleaves DFF-45, and, once caspase-activated deoxyribonuclease (CAD) is released, it can enter the nucleus, where it degrades chromosomal DNA to produce interchromosomal DNA fragmentation (*33*, *34*). We examined the cleavage of PARP and DFF-45 following the activation of caspase-3; COLO 205 cells were treated with various concentrations of shikonin for 12 h. Western blotting analysis showed that shikonin caused a dose-dependent proteolytic cleavage of PARP, with accumulation of 85-kDa species and concomitant disappearance of the full-size 116-kDa protein (**Figure 9A**). Higher shikonin concentrations yielded apparently the disappearance of PARP, possibly as a result of loss of cytoplasm by acute necrotic cell lysis. As shown in **Figure 9B** and **9C**, the cleavage of DFF-45 triggered by shikonin progressively increases in a dose- and time-dependent manner. These data are consistent with DNA fragmentation and caspase-3 activity. Taken together,

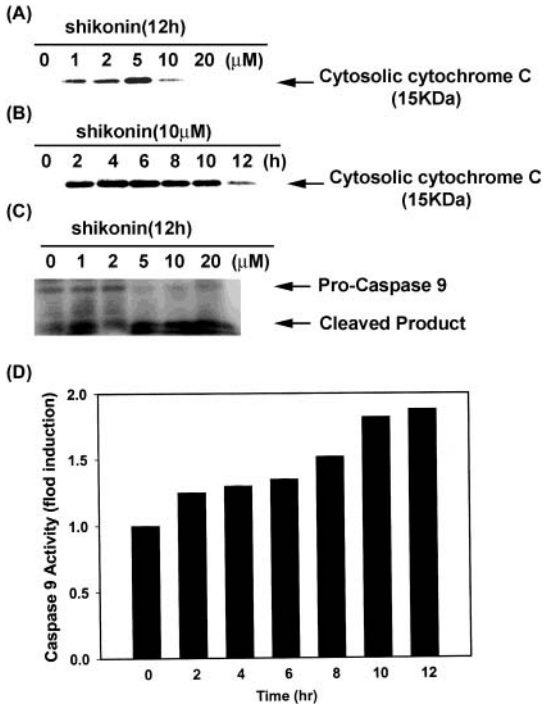

Figure 7. Induction of cytochrome *c* release and caspase 9 activation in shikonin induced apoptosis. (**A**) Cytochrome *c* was released from mitochondria into the cytosol. COLO 205 cells were treated with different concentrations of shikonin for 12 h and (**B**) 10 μM shikonin for different time periods or treated with 0.05% DMSO as vehicle control, and cytochrome *c* was detected by cytochrome *c* antibody. (**C**) Caspase-9 processing was induced by shikonin. (**D**) Caspase-9 activity was induced by 10 μM shikonin for 12 h, and detected as described under Materials and Methods. This experiment was repeated three times with similar results.

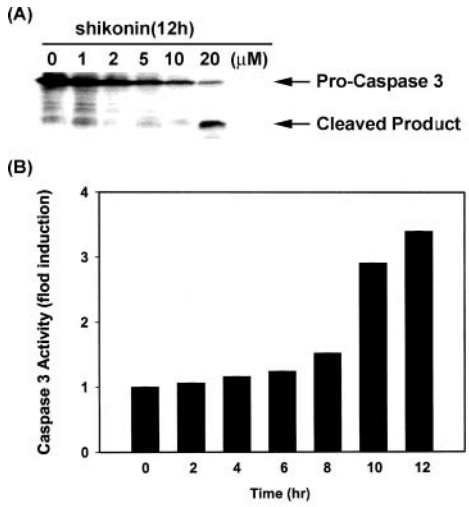

Figure 8. Cleavage and activation of caspase-3 during shikonin-induced apoptosis. (**A**) Cleavage of caspase-3 and (**B**) kinetics of caspase-3 activation. COLO 205 cells were treated with 10 μM shikonin for different time periods or treated with 0.05% DMSO as vehicle control. Western blot data presented are representative of those obtained in at least three separate experiments. Caspase activities were analyzed as described under Materials and Methods. This experiment was repeated three times with similar results.

our results suggested that the mechanism by which shikonin induced apoptosis involved the dissipation of $\Delta\Psi$m, resulting in cytochrome *c* release from mitochondria and subsequently activating the caspase cascades.

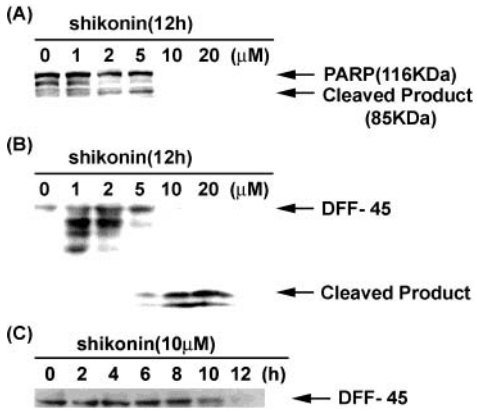

Figure 9. Cleavage of PARP and DFF-45 during shikonin-induced apoptosis. (**A**) Dose-dependent increases in the cleavage of PARP by shikonin. (**B**) COLO 205 cells treated with increasing doses of shikonin for 12 h and (**C**) time-dependent cleavage of DFF-45 by shikonin. Cells were treated as indicated and by western blotting as described under Materials and Methods. This experiment was repeated three times with similar results.

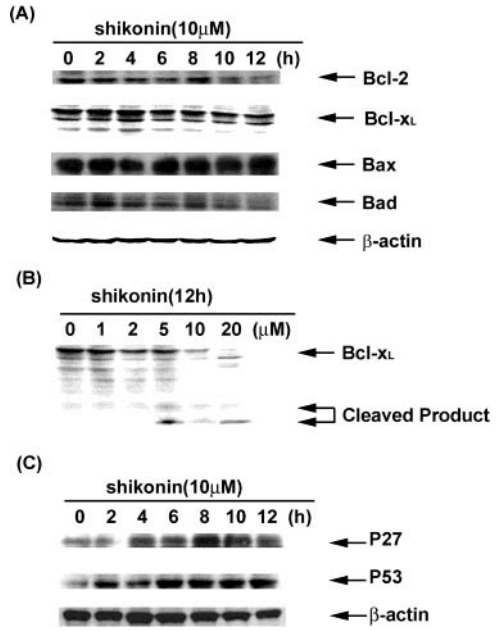

Figure 10. Western blot analysis for the expression of Bcl-2 protein family, p27, and p53 in shikonin-treated COLO 205 cells. Cells from each time point following 10 μM shikonin treatment were analyzed. (**A**) Expression of Bcl-2, Bcl-X_L, Bax, and Bad, respectively. (**B**) Dose-dependent increases in the cleavage of Bcl-X_L by shikonin. (**C**) Expression of p27 and p53. Western blotting analysis using specific antibodies against Bcl-2, Bcl-X_L, Bax, Bad, p27, and p53, respectively. This experiment was repeated three times with similar results.

Effect of Shikonin on the Expression of Bcl-2 Family, p27/Kip, and p53 Proteins in COLO 205 Cells. Several gene products are known to be important in controlling the apoptotic process. The imbalance of expression of anti- and pro-apoptotic proteins after the stimulus is one of the major mechanisms underlying the ultimate fate of cells in apoptotic process. To determine if shikonin has an effect on the expression levels of these gene products, a similar time-course experiment was performed and cellular lysates were analyzed by western blotting. As shown in **Figure 10A**, exposure of COLO 205 cells to 10 μm shikonin resulted in down-regulation of Bcl-2 and Bcl-X_L (**Figure 10A**, upper panel), and Bcl-X_L was cleaved to produce two small-size fragments (**Figure 10B**). In contrast, the Bad protein began to increase after 2 h of incubation (**Figure 10A**, lower panel). There was a slight increase of Bax expression (**Figure 10A**, middle panel) after shikonin treatment in COLO 205 cells. Previous studies demonstrated that the p27/Kip1 protein plays an important role in human cancer cell apoptosis induced by various stimuli (*35*, *36*). As shown in **Figure 10C**, shikonin markedly up-regulated the level of p27/Kip1 at 4 h after shikonin-treatment. Previous studies demonstrated that p53 protein is a potent transcription factor, activated and accumulated in response to DNA-damaging agents (*37*), leading to cell cycle arrest or apoptosis (*38*, *39*). In our results in **Figure 10B**, the p53 protein level was elevated significantly at 2 h after 10 μM shikonin treatment in COLO 205 cells. Such results implied that the p27/Kip and p53 might be playing some important roles in shikonin-induced apoptosis observed in the COLO 205 cells.

DISCUSSION

Shikonin, a red naphthoquinone derivative, found in most of the traditional medicinal plants of the Boraginaceae family, is the active principle of the medicinal plant *Lithospermum erythrorhizon* (*40*). Our recent report demonstrated that garcinol more strongly induced apoptosis than curcumin in the HL-60 cells (*26*). Shikonin is structurally related to garcinol, which contains both a β-diketone moiety and a prenyl moiety. The prenylated flavonois have been reported to be potential natural medicines (*41*−*43*). Shikonin has been reported to exhibit many biological effects including anticancer activity (*7*). Yet their anticancer mechanism is still elusive. In this study, we clarified the molecular mechanism by which shikonin triggered human colorectal carcinoma COLO 205 cells undergoing apoptosis. The present results demonstrate for the first time that shikonin can induce apoptosis in human colorectal carcinoma cells. The induction of apoptosis occurred within several hours, consistent with the view that shikonin induces apoptosis by activating the pre-existing apoptosis machinery. Indeed, treatment with shkonin caused an induction of caspase-3 activity and degradation of PARP and DFF-45, which precedes onset of apoptosis. Many lines of evidence demonstrated that activation of caspase is a central mechanism of apoptosis, and caspases are considered to be the "executioners" of cell death (*44*). Through these domains, the caspases interact with adaptor proteins that recruit them to specific "death complexes". In mammals, the death complexes include the Apaf-1/caspase-9 apoptosome and the FADD/caspase-8 death-inducing signaling complex (DISC). The apoptosome and DISC are thought to account for most caspase-dependent apoptosis. The upstream signaling pathways leading to assembly of the death complex are often called the mitochondrial (intrinsic) and receptor (extrinsic) pathways of apoptosis (*45*). However, we were unable to detect significant changes in the activities of caspase-1 and caspase-8 (data not shown) during shikonin treatment. This raises the possibility that factors or proteases other than caspase-1 and caspase-8 are involved in the activation of caspase-3. Caspase-3 is activated by two sequential proteolytic events that cleaved a 32-kDa precursor at aspartic acid residues to generate an active heterodimer of 20- and 12-kDa subunits (*46*). In fact, in vitro studies have previously identified Apaf1, cytochrome *c*, and caspase-9 as participants in a complex important for caspase-3 activation. In vitro depletion of caspase-9 from cytosolic fractions resulted in the failure of caspase-3 activation (*33*). Taken together, these data suggest a linear and specific activation cascade between caspase-9 and caspase-3 in response to cytochrome *c* release from mitochondria. Release of cytochrome *c* from the mitochondria has been shown to be an almost universal phenomenon during apoptosis, although it is unclear whether the cytochrome

c-mediated caspase cascade is triggered only by a few apoptotic stimuli, or serves as a general amplification mechanism to accelerate cell death (*47*). In this study, we found that shikonin induced the release of cytochrome *c* and the activation of caspase-9.

Recent studies have suggested that mitochondria play a pivotal role in apoptosis triggered by many stimuli (*48*, *49*). Herein, we demonstrated that shikonin could disrupt the function of mitochondria at the early stage of apoptosis and subsequently coordinate caspase activation through the release of cytochrome *c*. COLO 205 cells showed increased ROS production after shikonin treatment (**Figure 6**). This sequence suggested a possible role of ROS as mediator of the apoptotic response to shikonin. The increase in ROS was probably due to the affected mitochondria cycling dioxygen through the electron transport assembly, and generating ROS by one-electron-transfer mitochondria could be a main target of nonspecific damage through oxidative stress at the level of the outer and inner membranes (*50*, *51*). As a consequence of oxidative membrane damage, membrane potential and permeability-barrier function are impaired, leading to further mitochondrial damage. Recently, oxidative damage to the mitochondrial membrane due to increased generation of ROS has been shown to play a role in apoptosis (*52*). Mitochondria have also been implicated as a source of ROS during apoptosis. Reduced mitochondria membrane potential has recently been shown to lead to increased generation of ROS and apoptosis (*53*).

An important area of future research is identification of the genes that are involved in the apoptotic program of cell death. In fact, the finding that cell death occurs at a certain time and at certain locations during precise stages of normal development or metamorphosis implies that there are genes responsible for the occurrence of cell death. The best example of a cell death-associated gene is probably the bcl-2 gene (*22*). The Bcl-2 family proteins, whose members may be anti-apoptotic or pro-apoptotic, regulate cell death by controlling the mitochondria membrane permeability during apoptosis (*54*, *55*). However, the transfection studies have expressed that when cleaved by caspase, Bcl-2 and Bcl-X_L proteins are converted into potent pro-apoptotic factors, and they may accelerate apoptosis by amplifying the caspase cascade (*25*). Bad is a distant relative of Bcl-2 and acts to promote cell death (*56*). We, therefore, inferred that the Bcl-2 family protein might participate in the event that controlled the change in mitochondrial membrane potential and trigger cytochrome *c* release during apoptosis induced by shikonin. In our study, we found the down-regulation of the Bcl-2 expression and the concomitant up-regulation of the Bad expression under the same conditions, and the Bax protein level slightly increased in shikonin-treated cells (**Figure 10A**). We also found the cleavage of Bcl-X_L during shikonin-induced apoptosis in COLO 205 cells (**Figure 10B**). However, the ratio between Bcl-2 and Bax and Bcl-X_L cleavage determines cell survival or death. The p53 tumor suppressor is a predominant nuclear transcription factor, activated by various stresses including chemopreventive agents (*57*). Previous studies demonstrated that p27/Kip protein plays an important role in human cancer cell apoptosis and cell cycle arrest induced by various stimuli (*35*, *36*). Treatment of the COLO 205 cells with shikonin resulted in an increase in the level of p53 and p27/Kip protein (**Figure 10C**). The results presented herein might account for oncogenes that can facilitate cytochrome *c* release dependently of the p53 protein that affects mitochondrial function.

In summary, we have demonstrated that the cancer chemopreventive agent shikonin is able to induce apoptosis in a dose-dependent manner. These results show that shikonin is able to induce a loss of mitochondrial transmembrane potential and release of mitochondrial cytochrome *c* into the cytosol and correlated ROS generation after shikonin treatment. It also induces procaspase-9 processing, activates caspase-3, and produces the cleavage of PARP and DFF-45 and activation of endonuclease. Therefore, we speculate that the induction of apoptosis observed in this study may provide a distinct mechanism for the chemopreventive function of shikonin.

ABBREVIATIONS USED

DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; Apaf-1, apoptotic protease activating factor-1; ICAD, inhibitor of caspase-3-activated DNase DHE, dihydroethidium; ROS, reactive oxygen species.

LITERATURE CITED

(1) Kelloff, G. J.; Boone, C. W.; Crowell, J. A.; Steele, V. E.; Lubet, R.; Sigman, C. C. Chemopreventive drug development: perspectives and progress. *Cancer Epidemiol., Biomarkers Prev.* **1994**, *3*, 85−98.

(2) Kang, S. K.; Burnett, C. A.; Freund, E.; Walker, J.; Lalich, N.; Sestito, J. Gastrointestinal cancer mortality of workers in occupations with high asbestos exposures. *Am. J. Ind. Med.* **1997**, *31*, 713−718.

(3) Hirota, S. Gastrointestinal stromal tumors: their origin and cause. *Int. J. Clin. Oncol.* **2001**, *6*, 1−5.

(4) Strickland, L.; Letson, G. D.; Muro-Cacho, C. A. Gastrointestinal stromal tumors. *Cancer Control* **2001**, *8*, 252−261.

(5) Sharma, S.; Stutzman, J. D.; Kelloff, G. J.; Steele, V. E. Screening of potential chemopreventive agents using biochemical markers of carcinogenesis. *Cancer Res.* **1994**, *54*, 5848−5855.

(6) Sankawa, U.; Ebizuka, Y.; Miyazaki, T.; Isomura, Y.; Otsuka, H. Antitumor activity of shikonin and its derivatives. *Chem. Pharm. Bull.* **1977**, *25*, 2392−2395.

(7) Sankawa, U.; Otsuka, H.; Kataoka, Y.; Iitaka, Y.; Hoshi, A.; Kuretani, K. Antitumor activity of shikonin, alkannin and their derivatives. II. X-ray analysis of cyclo-alkannin leucoacetate, tautomerism of alkannin and cyclo-alkannin and antitumor activity of alkannin derivatives. *Chem. Pharm. Bull.* **1981**, *29*, 116−122.

(8) Singh, B.; Sharma, M. K.; Meghwal, P. R.; Sahu, P. M.; Singh, S. Anti-inflammatory activity of shikonin derivatives from Arnebia hispidissima. *Phytomedicine* **2003**, *10*, 375−380.

(9) Shen, C. C.; Syu, W. J.; Li, S. Y.; Lin, C. H.; Lee, G. H.; Sun, C. M. Antimicrobial activities of naphthazarins from Arnebia euchroma. *J. Nat. Prod.* **2002**, *65*, 1857−1862.

(10) Gao, D.; Hiromura, M.; Yasui, H.; Sakurai, H. Direct reaction between shikonin and thiols induces apoptosis in HL60 cells. *Biol. Pharm. Bull.* **2002**, *25*, 827−832.

(11) Hashimoto, S.; Xu, M.; Masuda, Y.; Aiuchi, T.; Nakajo, S.; Cao, J.; Miyakoshi, M.; Ida, Y.; Nakaya, K. β-Hydroxyisovalerylshikonin inhibits the cell growth of various cancer cell lines and induces apoptosis in leukemia HL-60 cells through a mechanism different from those of Fas and etoposide. *J. Biochem.* **1999**, *125*, 17−23.

(12) Ozaki, Y.; Ohno, A.; Saito, Y.; Satake, M. Accelerative effect of shikonin, alkannin and acetylshikonin on the proliferation of granulation tissue in rats. *Biol. Pharm. Bull.* **1994**, *17*, 1075−1077.

(13) Wyllie, A. H.; Kerr, J. F.; Currie, A. R. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **1980**, *68*, 251−306.

(14) Boise, L. H.; Gonzalez-Garcia, M.; Postema, C. E.; Ding, L.; Lindsten, T.; Turka, L. A.; Mao, X.; Nunez, G.; Thompson, C. B. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **1993**, *74*, 597−608.

(15) Yang, X.; Stennicke, H. R.; Wang, B.; Green, D. R.; Janicke, R. U.; Srinivasan, A.; Seth, P.; Salvesen, G. S.; Froelich, C. J. Granzyme B mimics apical caspases. Description of a unified pathway for trans-activation of executioner caspase-3 and -7. *J. Biol. Chem.* **1998**, *273*, 34278−34283.

(16) Salvesen, G. S.; Dixit, V. M. Caspases: intracellular signaling by proteolysis. *Cell* **1997**, *91*, 443−446.
(17) Froelich, C. J.; Dixit, V. M.; Yang, X. Lymphocyte granule-mediated apoptosis: matters of viral mimicry and deadly proteases. *Immunol. Today* **1998**, *19*, 30−36.
(18) Ashkenazi, A.; Dixit, V. M. Death receptors: signaling and modulation. *Science* **1998**, *281*, 1305−1308.
(19) Li, P.; Nijhawan, D.; Budihardjo, I.; Srinivasula, S. M.; Ahmad, M.; Alnemri, E. S.; Wang, X. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **1997**, *91*, 479−489.
(20) Liu, X.; Kim, C. N.; Yang, J.; Jemmerson, R.; Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* **1996**, *86*, 147−157.
(21) Yang, J.; Liu, X.; Bhalla, K.; Kim, C. N.; Ibrado, A. M.; Cai, J.; Peng, T. I.; Jones, D. P.; Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science* **1997**, *275*, 1129−1132.
(22) Merry, D. E.; Korsmeyer, S. J. Bcl-2 gene family in the nervous system. *Annu. Rev. Neurosci.* **1997**, *20*, 245−267.
(23) Sato, T.; Hanada, M.; Bodrug, S.; Irie, S.; Iwama, N.; Boise, L. H.; Thompson, C. B.; Golemis, E.; Fong, L.; Wang, H. G. Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9238−9242.
(24) Kluck, R. M.; Bossy-Wetzel, E.; Green, D. R.; Newmeyer, D. D. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **1997**, *275*, 1132−1136.
(25) Bellows, D. S.; Chau, B. N.; Lee, P.; Lazebnik, Y.; Burns, W. H.; Hardwick, J. M. Antiapoptotic herpesvirus Bcl-2 homologs escape caspase-mediated conversion to proapoptotic proteins. *J. Virol.* **2000**, *74*, 5024−5031.
(26) Pan, M. H.; Chang, W. L.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. Induction of apoptosis by garcinol and curcumin through cytochrome *c* release and activation of caspases in human leukemia HL-60 cells. *J. Agric. Food Chem.* **2001**, *49*, 1464−1474.
(27) Telford, W. G.; King, L. E.; Fraker, P. J. Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry1. *Cytometry* **1992**, *13*, 137−143.
(28) Kroemer, G.; Reed, J. C. Mitochondrial control of cell death. *Nat. Med.* **2000**, *6*, 513−519.
(29) Bass, D. A.; Parce, J. W.; Dechatelet, L. R.; Szejda, P.; Seeds, M. C.; Thomas, M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol.* **1983**, *130*, 1910−1917.
(30) Nicholson, D. W.; Thornberry, N. A. Caspases: killer proteases. *Trends Biochem. Sci.* **1997**, *22*, 299−306.
(31) Thompson, C. B. Apoptosis in the pathogenesis and treatment of disease. *Science* **1995**, *267*, 1456−1462.
(32) Tewari, M.; Quan, L. T.; O'Rourke, K.; Desnoyers, S.; Zeng, Z.; Beidler, D. R.; Poirier, G. G.; Salvesen, G. S.; Dixit, V. M. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **1995**, *81*, 801−809.
(33) Liu, X.; Zou, H.; Slaughter, C.; Wang, X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **1997**, *89*, 175−184.
(34) Sakahira, H.; Enari, M.; Nagata, S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **1998**, *391*, 96−99.
(35) Huang, L.; Pardee, A. B. β-Lapachone induces cell cycle arrest and apoptosis in human colon cancer cells. *Mol. Med.* **1999**, *5*, 711−720.
(36) Fujieda, S.; Inuzuka, M.; Tanaka, N.; Sunaga, H.; Fan, G. K.; Ito, T.; Sugimoto, C.; Tsuzuki, H.; Saito, H. Expression of p27 is associated with Bax expression and spontaneous apoptosis in oral and oropharyngeal carcinoma. *Int. J. Cancer* **1999**, *84*, 315−320.
(37) Morgan, S. E.; Kastan, M. B. p53 and ATM: cell cycle, cell death, and cancer. *Adv. Cancer Res.* **1997**, *71*, 11−25.
(38) Ko, L. J.; Prives, C. p53: puzzle and paradigm. *Genes Dev.* **1996**, *10*, 1054−1072.
(39) Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell* **1997**, *88*, 323−331.
(40) Yazaki, K.; Kunihisa, M.; Fujisaki, T.; Sato, F. Geranyl diphosphate: 4-hydroxybenzoate geranyltransferase from Lithospermum erythrorhizon. Cloning and characterization of a ket enzyme in shikonin biosynthesis. *J. Biol. Chem.* **2002**, *277*, 6240−6246.
(41) Miranda, C. L.; Stevens, J. F.; Ivanov, V.; McCall, M.; Frei, B.; Deinzer, M. L.; Buhler, D. R. Antioxidant and prooxidant actions of prenylated and nonprenylated chalcones and flavanones in vitro. *J. Agric. Food Chem.* **2000**, *48*, 3876−3884.
(42) Henderson, M. C.; Miranda, C. L.; Stevens, J. F.; Deinzer, M. L.; Buhler, D. R. In vitro inhibition of human P450 enzymes by prenylated flavonoids from hops, Humulus lupulus. *Xenobiotica* **2000**, *30*, 235−251.
(43) Wang, B. H.; Ternai, B.; Polya, G. Specific inhibition of cyclic AMP-dependent protein kinase by warangalone and robustic acid. *Phytochemistry* **1997**, *44*, 787−796.
(44) Vaux, D. L.; Korsmeyer, S. J. Cell death in development. *Cell* **1999**, *96*, 245−254.
(45) Martin, S. J.; Green, D. R. Protease activation during apoptosis: death by a thousand cuts? *Cell* **1995**, *82*, 349−352.
(46) Nicholson, D. W.; Ali, A.; Thornberry, N. A.; Vaillancourt, J. P.; Ding, C. K.; Gallant, M.; Gareau, Y.; Griffin, P. R.; Labelle, M.; Lazebnik, Y. A. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **1995**, *376*, 37−43.
(47) Reed, J. C. Cytochrome *c*: can't live with it—can't live without it. *Cell* **1997**, *91*, 559−562.
(48) Desagher, S.; Martinou, J. C. Mitochondria as the central control point of apoptosis. *Trends Cell Biol.* **2000**, *10*, 369−377.
(49) Green, D. R.; Reed, J. C. Mitochondria and apoptosis. *Science* **1998**, *281*, 1309−1312.
(50) Buttke, T. M.; Sandstrom, P. A. Oxidative stress as a mediator of apoptosis. *Immunol. Today* **1994**, *15*, 7−10.
(51) Hockenbery, D. M.; Oltvai, Z. N.; Yin, X. M.; Milliman, C. L.; Korsmeyer, S. J. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **1993**, *75*, 241−251.
(52) Peled-Kamar, M.; Lotem, J.; Okon, E.; Sachs, L.; Groner, Y. Thymic abnormalities and enhanced apoptosis of thymocytes and bone marrow cells in transgenic mice overexpressing Cu/Zn-superoxide dismutase: implications for Down syndrome. *EMBO J.* **1995**, *14*, 4985−4993.
(53) Zamzami, N.; Marchetti, P.; Castedo, M.; Decaudin, D.; Macho, A.; Hirsch, T.; Susin, S. A.; Petit, P. X.; Mignotte, B.; Kroemer, G. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J. Exp. Med.* **1995**, *182*, 367−377.
(54) Adams, J. M.; Cory, S. The Bcl-2 protein family: arbiters of cell survival. *Science* **1998**, *281*, 1322−1326.
(55) Tsujimoto, Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells* **1998**, *3*, 697−707.
(56) Mok, C. L.; Gil-Gomez, G.; Williams, O.; Coles, M.; Taga, S.; Tolaini, M.; Norton, T.; Kioussis, D.; Brady, H. J. Bad can act as a key regulator of T cell apoptosis and T cell development. *J. Exp. Med.* **1999**, *189*, 575−586.
(57) Jimenez, G. S.; Khan, S. H.; Stommel, J. M.; Wahl, G. M. p53 regulation by post-translational modification and nuclear retention in response to diverse stresses. *Oncogene* **1999**, *18*, 7656−7665.

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