Curcumin-induced Cell Cycle Arrest and Apoptosis in Human Acute Promyelocytic Leukemia HL-60 Cells *via* MMP Changes and Caspase-3 Activation

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Abstract. *Curcumin (diferuloylmethane), is a natural product derived from the root of the plant Curcuma longa. For centuries, it has been used as a spice and as a herbal medicine in Chinese populations. Curcumin has been shown to inhibit cell proliferation, cell cycle arrest, cyclooxygenase (COX)-1 and -2 expression and apoptosis in several human cancer cell lines. The aim of this investigation was to clarify the mechanisms by which curcumin induced cytotoxicity and apoptosis in human leukemia HL-60 cells. The effects of curcumin on the levels of reactive oxygen species (ROS), Ca^{+2} production, cyclin E, cdc25c, wee1, Bcl-2, Bax, the changes of mitochondrial membrane potential (MMP), cytochrome c release and the activation of caspase-3 were also investigated in the HL-60 cells. Results of flow cytometry and DAPI staining assays indicated that curcumin induced cytotoxicity and apoptosis in the examined cells. The results from flow cytometry assay indicated that curcumin induced ROS and Ca^{+2} productions, decreased the levels of MMP and increased the activity of caspase-3, leading to cell apoptosis. Western blot assay also revealed that curcumin increased the levels of Bax and the release of cytochrome c, and decreased the levels of Bcl-2 in the examined cells. The inhibition of caspase-3 activation by z-VAD-fmk (broad-spectrum caspase inhibitor) completely blocked curcumin-induced apoptosis in HL-60 cells.*

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Key Words: Curcumin, cell cycle arrest, apoptosis, caspase-3, HL-60.

Antitumor agents have been shown to cause drug-induced cellular lesions through cytotoxic mechanisms; some agents can trigger apoptosis, an irreversible process leading to the rapid elimination of tumor cells (1-4). The characteristics of apoptosis include membrane blebbing, cytoplasmic shrinkage, chromatin condensation, DNA fragmentation, loss of mitochondrial membrane potential (MMP) and the exposure of phosphatidylserine at the surface of the cells (5-13). The activation of caspase-3 is required for apoptosis, either by the caspase-3 or -9 pathway. The modulated expression of cell cycle regulatory molecules in cell cycle arrest or apoptosis have been shown in many cell types (14-16). The effect of curcumin on the cell cycle and on the activation of caspase-3 in human HL-60 leukemia cells was investigated here.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), a polyphenol derived from the plant *Curcuma longa*, is considered a promising anticancer drug due to its efficient induction of proliferation arrest and apoptosis in a variety of tumor cells (17-22). Curcumin exhibits pro-oxidant properties under certain conditions (22-26), such as high concentrations (*e.g.,* 50 mM), which were found to promote reactive oxygen species (ROS) generation (22-24), while a low concentration of curcumin (*e.g.,* 10 mM) reduced ROS generation (27, 28). Both the antioxidant and pro-oxidant activities of curcumin are considered to be involved in anticancer activity (17, 21, 22, 29). Our previous study have shown that curcumin induced apoptosis *via* ROS in human colon cancer colo 205 cells (30). It has also been reported that curcumin exerts anticancer activity in human leukemia cells by reducing ROS generation at low concentrations and enhances ROS generation at high levels (31). However, no reports address the effects of curcumin on the induction of apoptosis in human leukemia cells with regard to calcium production

and the changes of the MMP. Therefore, the effects of curcumin on the molecular signaling pathway, which leads to cell cycle arrest and apoptosis in human leukemia HL-60 cells, were investigated.

Materials and Methods

Chemicals and reagents. Curcumin, propidium iodide (PI), ribonuclease-A, Tris-HCl, triton X-100 and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), TE buffer and potassium phosphates were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA). The ROS, Ca^{2+} and caspase-3 activity assay kit was from Merck (Merck Ltd, Taiwan).

Leukemia cell line (HL-60). The human HL-60 leukemia cell line 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 in humidified 5% CO_2 and 95% air atmosphere in RPMI 1640 medium supplemented with 10% FBS, 1% glutamine and penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin). All data presented in this report are from at least 3 independent experiments (30, 32).

Curcumin-induced morphological changes in HL-60 cells as examined with a contrast-phase microscope. The HL-60 cells were plated in 12-well plates at a density of $5x10^3$ cells/well and were grown for 24 h. The various concentrations of curcumin (0, 0.1, 0.5, 1, 5 and 10 μM) were then added to the cultures for various time periods of incubation. The cells were moved to the plate and were then examined by a contrast-phase microscope and photographed (30, 32).

Curcumin effect on cell viability as determined by flow cytometry. The HL-60 cells were plated in 12-well plates at a density of $5x10^5$ cells/well and were grown for 24 h. The various concentrations of curcumin (0, 1, 5 and 10 μM) were added for various time periods of incubation. DMSO (solvent) was used for the control regimen. To determine cell viability, the flow cytometric protocol was used, as previously described (30, 32).

Curcumin effect on cell cycle and apoptosis in HL-60 cells as examined by flow cytometry. Approximately $5x10^5$ HL-60 cells/well in 12-well plates were incubated with various concentrations of curcumin (0, 0.1, 0.5, 1, 5 and 10 μM) for various time periods. The cells were harvested and fixed gently (drop by drop) in 70% ethanol (PBS) and kept at 4˚C overnight. The cells were then resuspended in PBS containing 40 μg/ml PI, 0.1 mg/ml RNase (Sigma) and 0.1% Triton X-100. After 30 min at 37˚C in the dark, the cells were analyzed with a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm (32). The percentage of cells that had undergone apoptosis was assessed as the ratio of the fluorescent area (sub-G1) less than the G0/G1 peak to the total area of fluorescence (30, 32).

Curcumin-induced apoptosis of HL-60 cells as examined by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. The cells ($5x10^4$ cells/ml) were treated with or without various concentrations of curcumin (0, 1, 5 and 10 μM) for 48 h and were then isolated for DAPI staining. After treatment, the cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and were stained with 1 μg/ml DAPI for 5 min at 37˚C. The cells were then washed with PBS and examined by fluorescence microscopy (Nikon, 200X) (30, 32).

Curcumin effect on ROS levels in HL-60 cells as examined by flow cytometry. The ROS levels in the HL-60 cells were examined by flow cytometry, using 2,7-dichlorodihydrofluorescein diacetate. The cells ($5x10^5$ cells/ml) were treated with or without various concentrations of curcumin (0, 1, 5 and 10 μM) for 24 h. The cells harvested and washed twice, re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) (DCFH-DA, Sigma) and incubated at 37˚C for 30 min. The detected changes of ROS were then analyzed by flow cytometry (Becton Dickinson FACS Calibur) (14, 30).

Curcumin effect on Ca^{2+} levels in HL-60 cells as examined by flow cytometry. The cells ($5x10^5$ cells/ml) were treated with or without various concentrations of curcumin (0, 0.1, 1, 5 and 10 μM) for 24 h before being isolated for detection of the changes in Ca^{+2} concentration. The cells were harvested and washed twice, once for apoptosis analysis and the other for re-suspension in Indo 1/AM (3 μg/ml) (Calbiochem, La Jolla, CA, USA), incubated at 37˚C for 30 min and analyzed by flow cytometry (Becton Dickinson FACS Calibur) (30, 33).

Curcumin effect on MMP levels in HL-60 cells as examined by flow cytometry. The cells *(*approximately $5x10^5$ cells/ml) were treated with or without various concentrations of curcumin (0, 0.1, 1, 5 and 10 μM) for 24 h to detect the changes in the MMP. The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 mol/L), incubated at 37˚C for 30 min and analyzed by flow cytometry (Becton Dickinson FACS Calibur) (30, 34).

Curcumin effect on the caspase-3 activity in HL-60 cells as examined by flow cytometry. The cells *(*approximately $5x10^5$ cells/ml) were treated with or without various concentrations of curcumin (0, 1, 5 and 10 μM) for 24 h to detect caspase-3 activity. The cells were harvested and washed twice, re-suspended in 25 μl of Phiphiliux green (OncoImmunin, Inc. MD, USA), incubated at 37˚C for 60 min and analyzed by flow cytometry (Becton Dickinson FACS Calibur) (30, 34).

The caspase-3 inhibitor z-DEVD-fmk inhibition of curcumin-induced apoptosis in HL-60 cells. Whether or not caspase-3 activation was involved in the apoptosis triggered by curcumin was investigated. The HL-60 cells were pretreated with the caspase-3 inhibitor (z-DEVD-fmk) 3 h prior to treatment with 10 μM curcumin, followed by the examination of apoptosis and caspase-3 activity, as described above (30, 32).

Curcumin effect on cyclin E, cdc25c, wee1, Bax, Bcl-2 and cytochrome c levels in HL-60 cells as examined by Western blotting. Approximately $3x10^6$ cells/well in 12-well plates were incubated with various concentrations of curcumin (0, 1, 5 and 10 μM) for 24 h before the cells were harvested by centrifugation. Protein was extracted as previously described (32, 35). The cyclin E, cdc25c, wee1, Bax, Bcl-2 and cytochrome c levels were measured by sodium

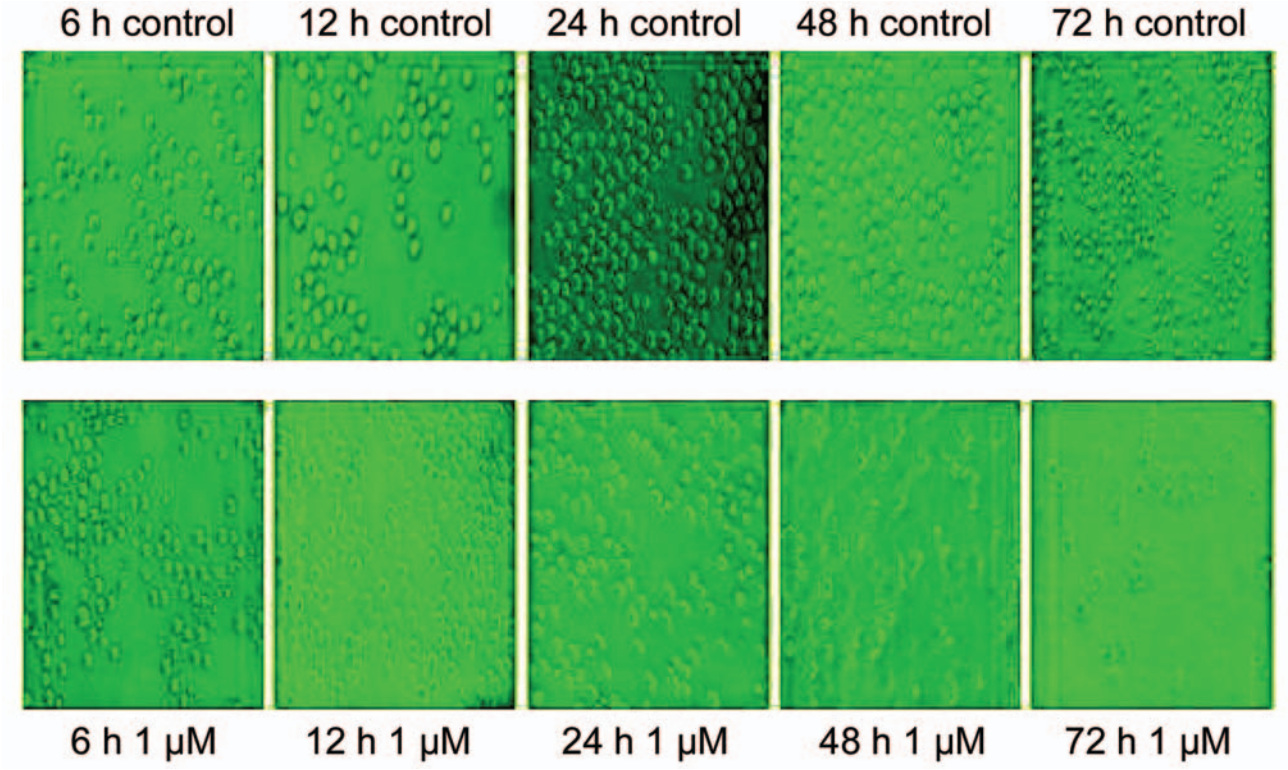


Figure 1. *Morphological changes of HL-60 cells in response to curcumin. HL-60 cells were treated with 1 μM curcumin for 6, 12, 24, 48 and 72 h. The cells were examined under a contrast-phase microscope and photographed.*

dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as described previously (32, 35).

Statistical analysis. The Student's *t*-test was used to analyze the statistical significance between the curcumin and control groups.

Results

Curcumin effect on cell morphology and viability. In the presence of curcumin (0, 0.1, 0.05, 1, 5 and 10 μM), the cells were photographed with a contrast-phase microscope, collected for staining by propidium iodine and analyzed for viability by flow cytometry. The results indicated that curcumin induced cell death. Increasing the concentration of curcumin resulted in increased morphological changes and a greater decrease in the number of viable cells (Figures 1 and 2).

Curcumin induced cell cycle arrest and apoptosis in HL-60 cells. The curcumin-treated cells showed a G0/G1-phase arrest, containing a sub-G0/G1-phase (corresponding to apoptotic cells) as shown in Figure 3A and B. A sub-G0/G1 apoptotic peak was very clear after the cells were treated for 48 h. The percentages of apoptotic cells after treatment with 1 μM curcumin for various time periods are shown in Figure 3C and D.

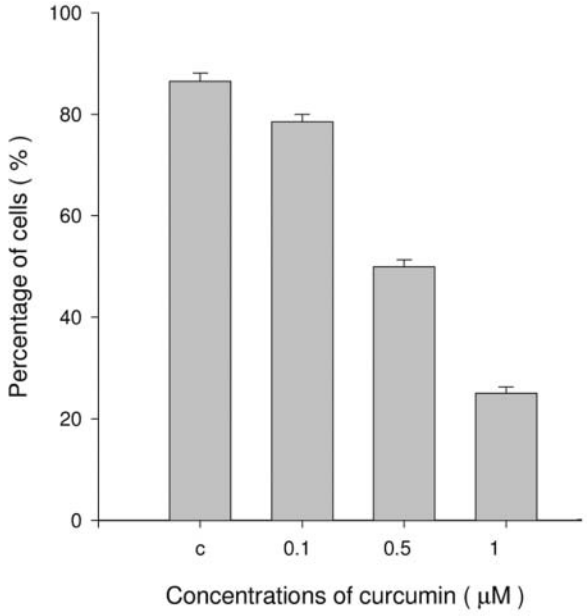


Figure 2. *Percentage of viable HL-60 cells in response to curcumin. HL-60 cells ($5x10^5$ cells/well; 12-well plates) were cultured in RPMI 1640 medium + 10% FBS with various concentrations of curcumin (0, 0.1, 0.5, 1, 5 and 10 μM) for 24 h. Then cells were collected and viable cell determinations were carried out by flow cytometry, as described in the Materials and Methods. Data represent mean±S.D. of 3 experiments.*

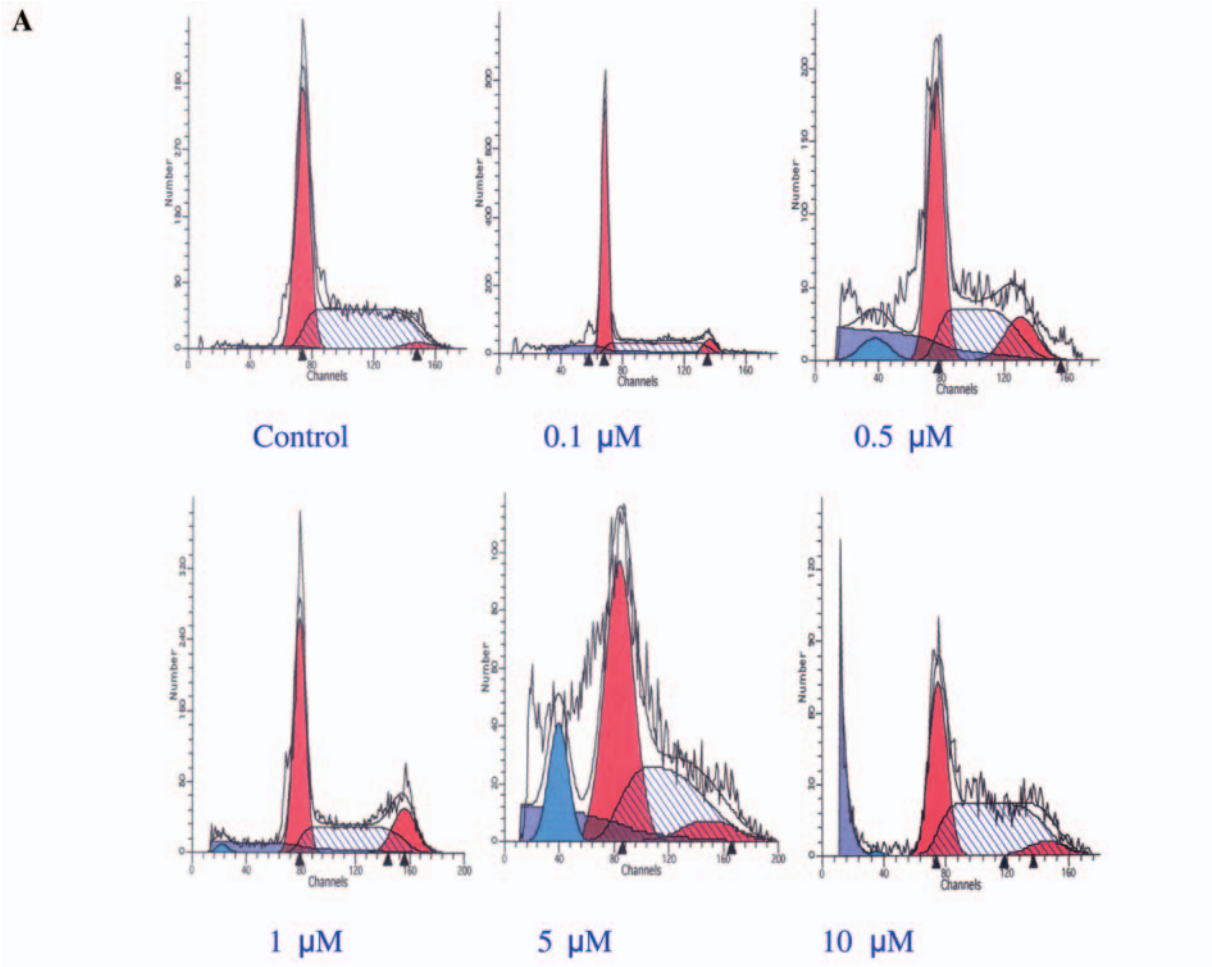


Figure 3. *continued*

Curcumin induced apoptosis in HL-60 cells as examined by DAPI staining. The number of HL-60 cells stained by DAPI was significantly different between curcumin-treated and control group. Increasing the dose of curcumin led to an increase in the DAPI staining in HL-60 cells (Figure 4), indicating that curcumin induced apoptosis in the examined cells.

Curcumin effect on the production of ROS. The percentage of cells stained by DCFH-DA (ROS levels) was significantly different between the curcumin-treated and control group. Increasing the curcumin incubation time led to an increase in ROS production in the examined cells (Table I).

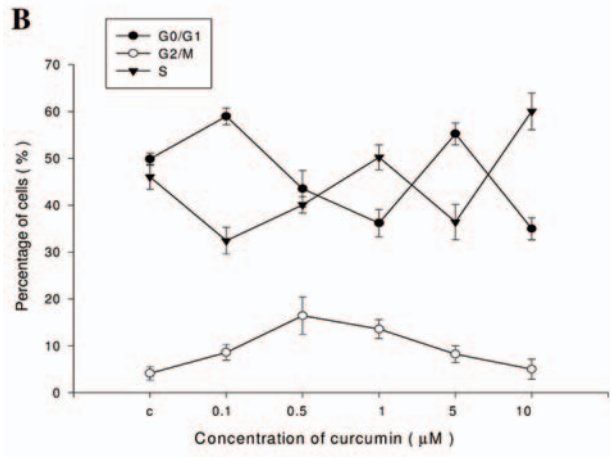
Curcumin effect on Ca^{+2} production. The percentage of Ca^{+2} cells stained by Indo 1/AM was significantly different between the curcumin-treated and control group. Increasing the dose of curcumin led to an increase in Ca^{+2} in the examined cells (Table II).

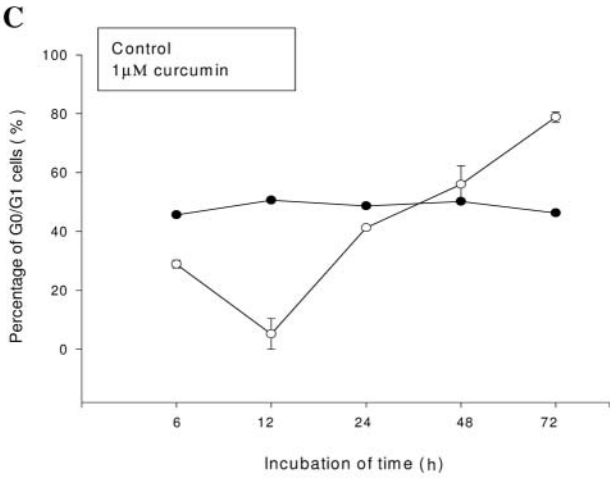
Curcumin effect on MMP levels. The MMP levels, as indicated by $DiOC_6$ staining were significantly different between the curcumin-treated and control group. Increasing the dose of curcumin led to a decrease in the MMP levels in the examined cells (Table III).

Caspase inhibitor (z-VAD-fmk) effect on curcumin-induced caspase-3 activity and apoptosis. The data presented in Figure 5A and B indicate that curcumin increased caspase-3 activity and that the caspase inhibitor z-VAD-fmk decreased caspase-3 activity in the HL-60 cells. The co-treatment of z-VAD-fmk and curcumin led to inhibition of curcumin-mediated caspase-3 activation accompanied by the marked attenuation of curcumin-induced apoptosis.

Curcumin effect on cyclin E, cdc25c, wee1, Bax, Bcl-2 and cytochrome c levels. The results from Western blotting are presented in Figure 6. The results demonstrated that 1-10 μM curcumin increased p27, p53, Bax and caspase-3 levels

Figure 3. *continued*





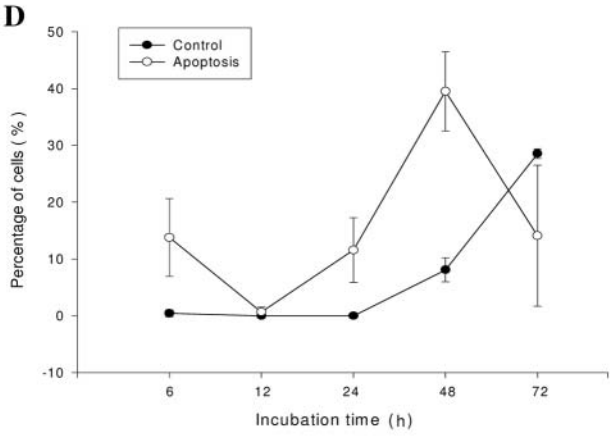


Figure 3. *Cell cycle phase and apoptosis in HL-60 cells in response to curcumin. HL-60 cells were cultured with varying concentrations of curcumin (0, 0.5, 1, 5 and 10 μM) for 24 h and the cells were then harvested and analyzed for cell cycle phase. (A) Representative profiles of flow cytometric analysis. (B) Dose-dependent effects. (C) Cells in G0/G1 phase and in the sub-G1 group. (D) Percent of apoptotic cells. The percent of apoptosis was evaluated by flow cytometry, as described in the Materials and Methods. Data represent mean±S.D. of 3 experiments. ●=G0/G1, ○=G2/M, ▼=S*

Table I. *Flow cytometric analysis of reactive oxygen species in HL-60 cells with or without 1 μM curcumin treatment.*

Incubation time (min)	Percentage of cells stained by DCFH-DA (% control)
0	1.4±0.6
15	12.6±2.1*
30	28.8±2.9*
60	41.1±3.8*
120	62.4±4.9*
240	74.8±6.7*

Values are mean±S.D. n=3. The HL-60 ($5x10^5$ cells/ml) were treated with 1 μM curcumin. The zero concentration was defined as control. The percentage of cells stained by DCFH-DA and the stained cells were determined by flow cytometry, as described in the Materials and Methods. *difference between curcumin-treated and control cells; $p<0.05$.

Table II. *Flow cytometric analysis of Ca^{+2} concentration in HL-60 cells with or without curcumin treatment.*

Curcumin (μM)	Percentage of cells stained by Indo-1/AM (% control)
0	0.8±0.3
0.1	3.1±0.7*
1	14.8±1.6*
5	32.4±2.9*
10	48.8±4.2*

Values are mean±S.D. n=3. The HL-60 cells ($5x10^5$ cells/ml) were treated with various concentrations of curcumin. The zero concentration was defined as control. The percentage of cells that were stained by Indo-1/AM and the stained cells were determined by flow cytometry, as described in the Materials and Methods section. *difference between curcumin-treated and control cells; $p<0.05$.

Table III. *Flow cytometric analysis of mitochondrial membrane potential in HL-60 with or without various concentrations of curcumin treatment for 24 h.*

Curcumin (μM)	Percentage of cells stained by $DiOC_6$
0 (control)	91.1±7.8
0.1	82.8±6.4
1	54.2±7.1*
5	36.3±4.8*
10	16.2±2.1*

Values are mean±S.D. n=3. The HL-60 cells ($5x10^5$ cells/ml) were treated with various concentrations of curcumin. The zero concentration was defined as control. The percentage of cells that were stained by $DiOC_6$, and the stained cells were determined by flow cytometry, as described in the Materials and Methods section. *difference between curcumin-treated and control cells; $p<0.05$.

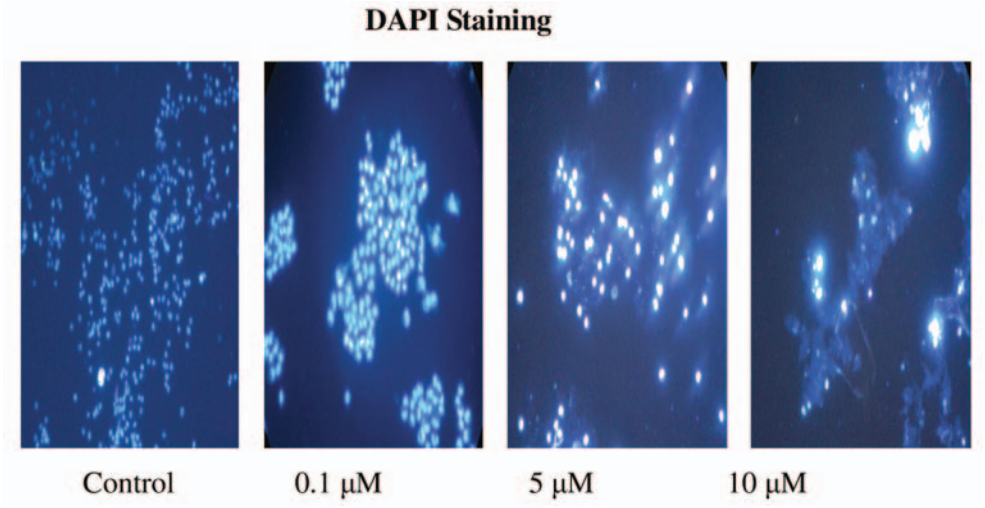


Figure 4. *DAPI staining for curcumin-induced apoptosis in HL-60 cells was examined by contrast-phase microscopy. The HL-60 cells were incubated with various concentrations of curcumin (0, 1, 5 and 10 μM) for 48 h. The cells were then harvested and analyzed for apoptosis by DAPI staining, as described in the Materials and Methods.*

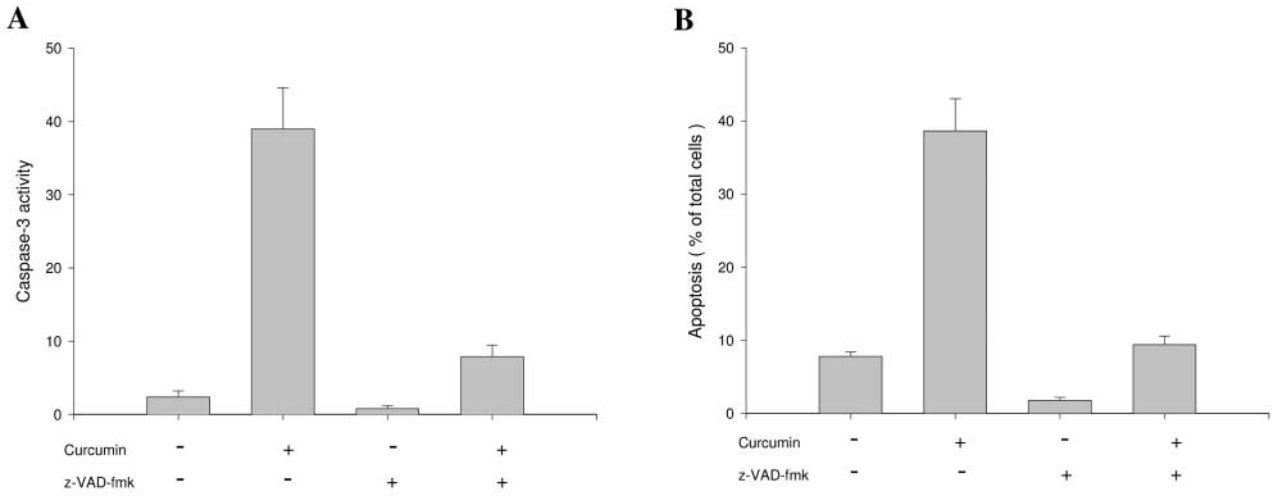
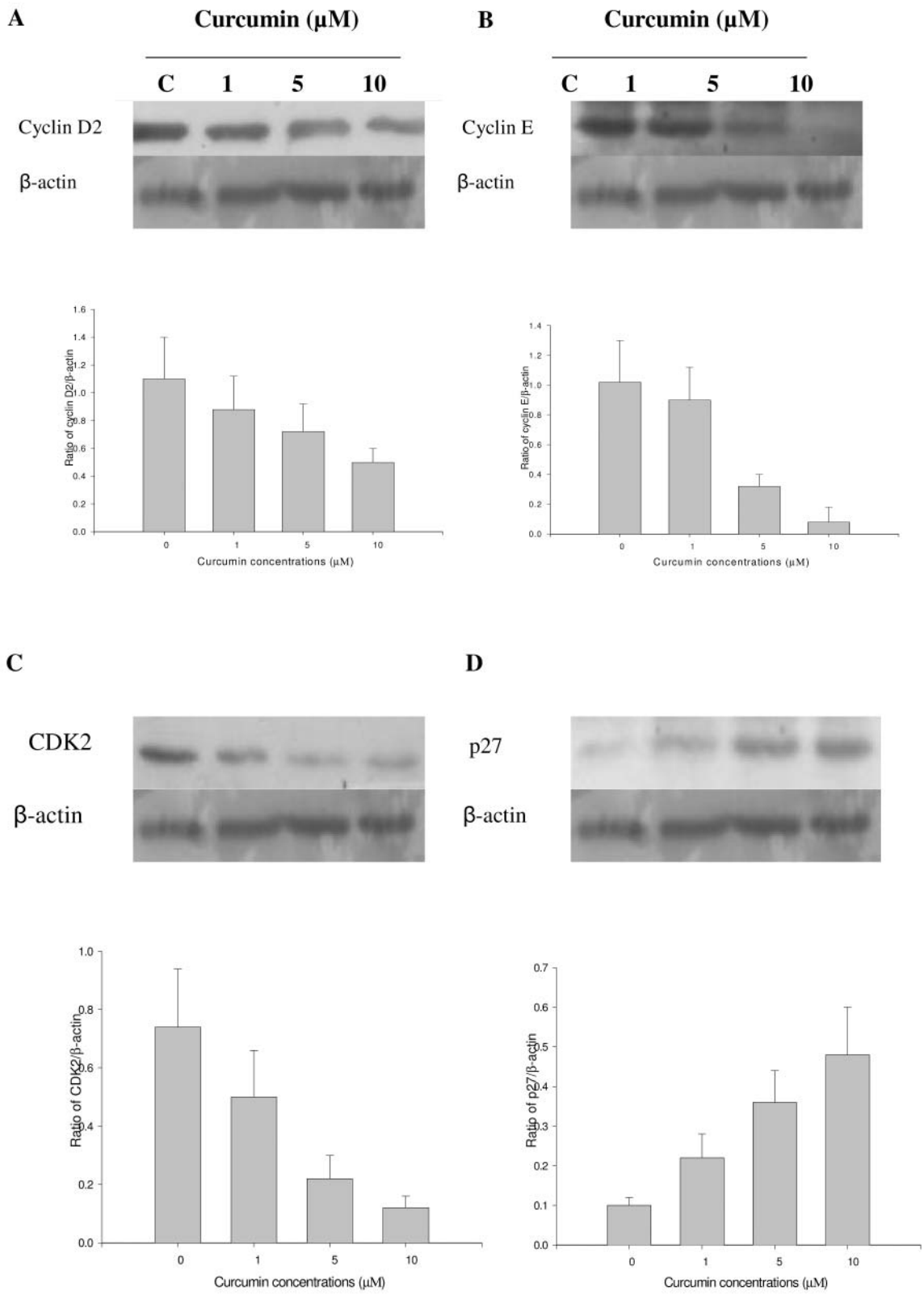


Figure 5. *Curcumin effects on caspase-3 activity and apoptosis in HL-60 cells were examined by flow cytometry. The HL-60 cells were incubated with 10 μM curcumin for various time periods. The cells were harvested and analyzed for caspase-3 activity (panel A) and apoptosis (panel B), as described in Materials and Methods. Data represent mean±S.D. of three experiments. *p<0.05.*

and decreased Bcl-2, Bcl-XL, CDK2, cyclin D2 and cyclin E levels in the examined cells in a dose-dependent manner. (Figure 6A and B).

Discussion

Many studies have shown that curcumin demonstrates anti-proliferation, anti-oxidant, pro-oxidant and antitumor activity in many human cell lines (22-29). Although it has been reported that curcumin induced apoptosis in human leukemia HL-60 cells (30), the exact pathway that led to apoptosis is still unclear. ROS is involved in the apoptosis induced by curcumin in HL-60 cells (30), as confirmed by our data as well. Numerous studies showed that ROS play an important role in the control of a variety of cell functions, such as proliferation, differentiation and apoptosis, and that the regulation of gene transcription is critically involved in these processes (36, 37). Sauer *et al.* also showed that ROS may regulate gene transcription by affecting the activation of transcription factors, such as nuclear factor κB (NF-κB) and the DNA binding activity of certain transcription factors, such as activator protein 1



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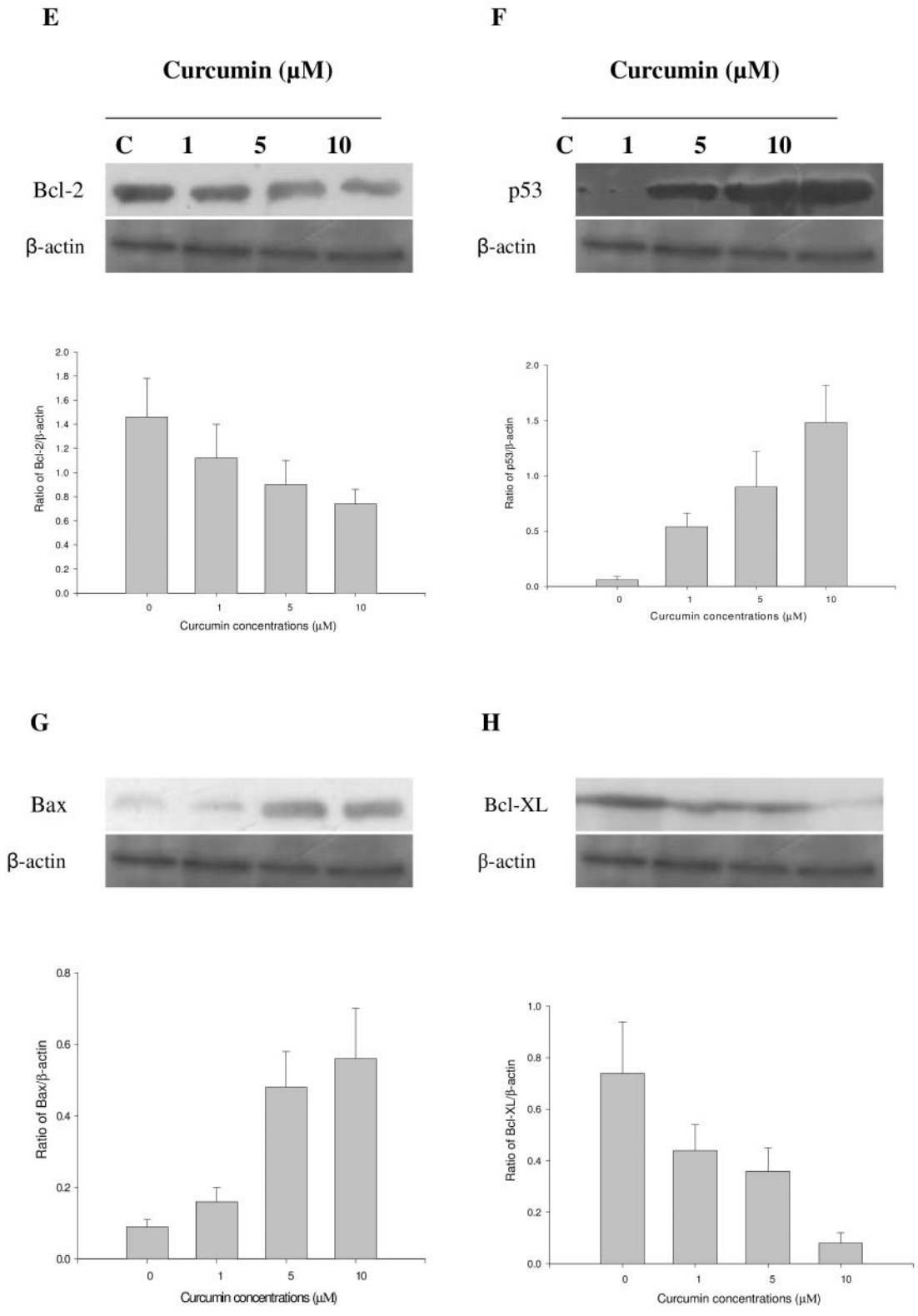

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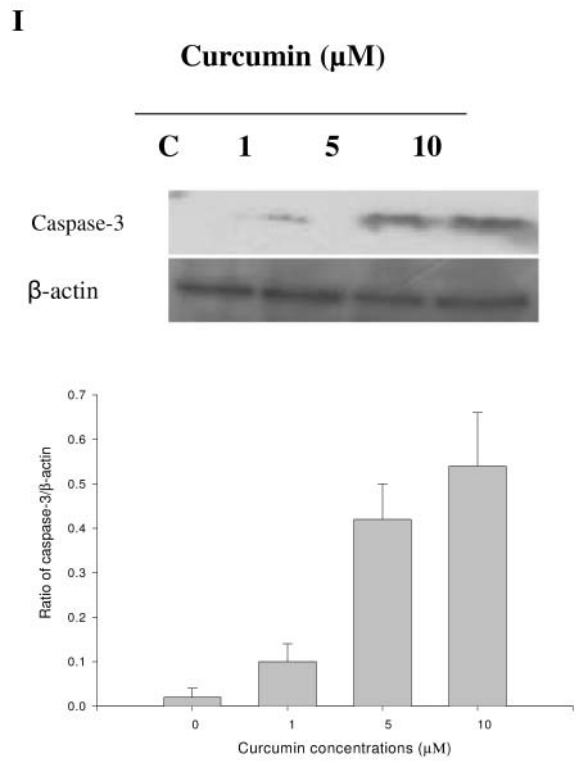

Figure 6. *Representative Western blots showing changes in the levels of cyclin E, cdc25c, wee1, Bcl-2, Bax and cytochrome c in HL-60 cells in response to curcumin treatment. HL-60 cells (5x10^6/ml) were treated with 0, 1, 5 and 10 μM curcumin for 24 h before cytosolic fraction and total protein were prepared and determined by Western blotting, as described in Materials and Methods.*

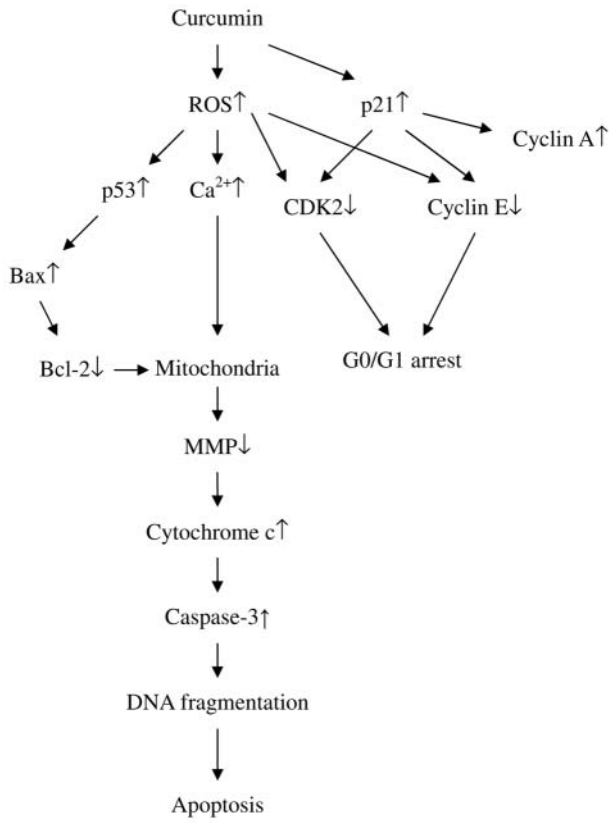

Figure 7. *Proposed flow chart for the curcumin mechanism of action on cell cyle arrest and apoptosis in HL-60 cells. Curcumin induced ROS and Ca^{+2} production, promoted Bax cdc25c and wee1 production, but decreased the expression of Bcl-2 and cyclin E as well as of MMP levels, causing the release of cytochrome c, leading to caspase-3 activity and finally apoptosis in the HL-60 cells.*

(AP-1) and p53 (38). Other investigators have also reported that curcumin affects the expression of NF-κB (30). It has been reported that high ROS levels induce, not only cell death, but also DNA damage and genomic instability (39), which in turn leads to tumorigenesis.

Our data indicate that curcumin induced apoptosis in HL-60 cells in a dose-dependent manner. Apoptosis is a programmed cell death *via* the expression and translocation of the Bcl-2 family proteins, changes of the mitochondrial membrane potential and the release of cytochrome c from mitochondria and activation of caspases to cause DNA fragmentation. We found that curcumin promoted Bax expression and the release of cytochrome c and DNA damage, and also promoted the activation of caspase-3 and inhibited the levels of Bcl-2 in the examined cells. In some cell lines, the overexpression of Bcl-2 was shown to protect the cells from apoptosis. Two pathways for the caspase cascade lead to the activation of caspase-3 for the occurrence of apoptosis, one involving caspase-8 and the other involving caspase-9 (40, 41). The Bcl-2 family proteins, including Bax, Bak, Bcl-2 and Bcl-XL, are well characterized regulators of apoptosis and are associated with mitochondria (41, 42). Moreover, the increased expression of caspase-3 activity in association with the decrease of the levels of MMP and Bcl-2 suggests that curcumin-induced apoptosis is mitochondria-dependent and signaling pathways may be involved in regulating caspase-3 activity, since the caspase inhibitor z-VAD-fmk led to a decrease in the caspase-3 activity and apoptosis.

Our data also indicate that curcumin induced G2/M arrest in HL-60 cells and this effect was also dose-dependent. Much evidence has shown that cyclins and cyclin-dependent kinases (cdks) are essential for cell cycle control and distinct pairs of cyclins and cdks regulate progression through different stages of the cell cycle.

Therefore, the molecular mechanisms of curcumin-induced G2/M arrest in human HL-60 cells was investigated. Curcumin promoted the expression of wee1 and cdc25c, inhibited cyclin B1, but did not affect cyclin E which may be the reason for curcumin-induced G2/M arrest. The transition process from G2- to M-phases is controlled by cyclin B1 (42-45). Cyclin B1 and phosphorylated Cdc2 are able to accelerate cyclin B1/cdc2 translocation into the nucleus and cell cycle regulation (46).

In conclusion, we present a working model (Figure 7) of our findings by which curcumin promoted ROS production and Ca^{2+} release, increased accumulation of Bax, but decreased the expression of Bcl-2 and led to the depolarization of MMP, thus, further enhancing the release of cytochrome c and increased caspase-3 activity before leading to apoptosis. Curcumin promoted the levels of wee1 and cdc25c, but inhibited the levels of cyclin E that led to G2/M arrest in HL-60 cells. The results of *in vitro* studies suggest the potential application of curcumin in the treatment of leukemia.

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Received February 10, 2006
Revised August 3, 2006
Accepted September 4, 2006