Morin Inhibits the Growth of Human Leukemia HL-60 Cells *via* Cell Cycle Arrest and Induction of Apoptosis through Mitochondria Dependent Pathway

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Abstract. *The effects of morin (3,5,7,20,40-pentahydroxyflavone) on human leukemia HL-60 cells in vitro were investigated and the molecular mechanisms of morin-induced G_2/M arrest and apoptosis in HL-60 cells were examined. Morin induced morphological changes and decreased the percentage of viable cells via induction of G_2/M-phase arrest and apoptosis. Morin-induced G_2/M-phase arrest was accompanied by the promotion of p21 and Wee1, and decreased levels of Cdc25c and cyclins A and B1 complex. Morin-induced apoptosis in HL-60 cells was also confirmed by flow cytometric assay, DNA gel electrophoresis for DNA fragmentation and DAPI staining. Morin induced apoptosis in time- and dose-dependent manners. Morin-induced apoptosis was associated with elevated intracellular reactive oxygen species (ROS) increased and Ca^{2+} production; decreased the levels of mitochondria membrane potential ($\Delta\Psi_m$) and increased caspase-3 activation. Collectively, these results suggest that the morin-induced apoptosis in HL-60 cells may result from the activation of caspase-3 and intracellular Ca^{2+} release, as well as the mitochondria membrane potential pathway.*

It is well known that high levels of reactive oxygen species (ROS) induce cell death (1), DNA damage and genomic instability which leads to tumorigenesis (2, 3). Cyclin-dependent kinases (CDKs) play an important role in the cell cycle. It is well known that many compounds affect cyclin and/or CDKs and, thus, may lead to cell cycle arrest (3). If a chemical causes DNA damage, the cell will undergo apoptosis. Apoptosis is a highly regulated cell death process characterized by ultrastructural modifications (nuclear alterations, caspase activation and DNA fragmentation) leading to the formation of apoptotic bodies that are phagocytosed by macrophages (4).

Numerous naturally occurring substances have been reported to exert their anticancer activity by blocking cell cycle progression and triggering tumor cell apoptosis. So far, the induction of apoptosis seems to be the best strategy for killing cancer cells. Therefore, cell cycle arrest and the induction of apoptosis in tumor cells has become the major indicator of effectiveness of anticancer agents (5, 6).

Morin (3,5,7,20,40-pentahydroxyflavone), a member of flavonols, is a yellowish pigment present in old fustic (*Chlorophora tinctoria*) and osage orange (*Maclura pomifera*) (7). It has been reported that morin hydrate (20 μM) inhibited caspase-3 activity (8). Morin (50 and 75 μmol/L) significantly inhibited cell proliferation compared with the control in LNCaP cells but the apoptotic effect only appeared at 72 h (9). In another study, morin (100 μM) inhibition of cell proliferation was less than 20% (10). It has been reported that morin (20 μM) inhibits growth of oral carcinomas more effectively than normal oral mucosa cells (11). Morin was a potent anti-hepatocellular transformation agent that inhibited cellular transformation by suppressing AP-1 activity and inducing S-phase arrest in human hepatocytes (12). However, there is no available information of the effect of morin on human leukemia HL-60 cells. Therefore, in the present study, the molecular mechanism of morin-induced cell cycle arrest and apoptosis in human leukemia HL-60 cells was investigated.

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Key Words: Morin, G_2/M-phase arrest, apoptosis, Ca^{2+} release, DAPI staining.

Materials and Methods

Materials. Morin, trypan blue, ribonuclease-A, propidium iodide (PI), Triton X-100 and Tris-HCl 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). RPMI-1640 medium, glutamine, fetal bovine serum (FBS) and penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Human leukemia HL-60 cell line. Human leukemia HL-60 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The HL-60 cells were placed into 75 cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 atmosphere in 90% RPMI 1640 medium with 2 mM L-gultamine adjusted to contain 1.5 g/L sodium bicarbonate, 10% FBS and 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin).

Morphological changes and viability of HL-60 cells treated with or without morin. HL-60 cells were plated in 12-well plates at a density of $2x10^5$ cells/well and grown for 24 h. Various concentrations of morin were then added to a final concentration of 0, 100, 200, 300, 400 and 500 μM, 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. The cells on the plates were photographed by light-phase microscope for examination of morphological changes, and the flow cytometric assay was used for cell viability examinations, as previously described (13).

Cell cycle and apoptosis from HL-60 cells treated with morin. Approximately $5x10^5$ cells/well of HL-60 cells with concentrations of 0, 100, 200, 300, 400 and 500 μM of morin were incubated in a 12-well plate for different time-periods. Cells were harvested by centrifugation and were fixed gently (drop by drop) with 70% ethanol (in PBS) kept at 4˚C overnight and then re-suspended in PBS containing 40 μg/mL PI, 0.1 mg/mL RNase and 0.1% Triton X-100 in a dark room. After 30 min at 37˚C, the cells were analyzed by flow-cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argonion laser at 488 nm wave-length. Apoptotic cells (sub-G_1-phase of the cell cycle) were quantified by Annexin V-FITC and propidium iodide double staining using a staining kit from PharMingen (San Diego, CA, USA) (13).

The effect of morin on caspase-3 activity of HL-60 cells examined by flow cytometry. Approximately $5x10^5$ cells/well of Hl-60 cells with concentrations of 0, 100, 200, 300, 400 and 500 μM of morin in a 12-well plates were incubated for 24 h. Cells were harvested by centrifugation and 50 μL of 10 μM caspase-3 substrate solution (PhiPhilux) (OncoImmunin, Inc., Gaithersburg, MD, USA) was added to the cell pellet ($1x10^5$ cells per sample) and incubated at 37˚C for 60 min. A substrate of PhiPhiLux which can penetrate into the cell nucleus, is converted to the fluorescent form when it is cleaved by the protease activity of caspase-3. The cells were washed once using 1 mL of ice cold PBS and re-suspended in a further 1 mL. Cells were analyzed by flow-cytometry as above. Then the caspase-3 activity was determined and analyzed (14).

The effect of morin on reactive oxygen species (ROS) in Hl-60 cells examined by flow cytometry. Approximately $5x10^5$ cells/well of HL-60 cells with 0, 100, 200, 300, 400 and 500 μM morin were incubated in a 12-well plate for 24 h to detect the changes of ROS. The cells were harvested and washed twice, re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) (DCFH-DA, Sigma), incubated at 37˚C for 30 min and analyzed by flow cytometry (15, 16). The % of cells being stained by DCFH-DA was calculated.

The effect of morin on Ca^{2+} concentrations in HL-60 cells examined by flow cytometry. The level of Ca^{2+} in HL-60 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using the Indo 1/AM (Calbiochem; La Jolla, CA). Approximately $2x10^5$ HL-60 cells/well with morin (0, 100, 200, 300, 400 and 500 μM) were incubated in a 12-well plate for 24 h to detect the changes of Ca^{+2} concentration. The cells were harvested and washed twice, re-suspended in 1-[2-amino-5-(6-carboxyindol-2-yl) phenoxy]-2-(2'-amino-5'-methylphenoxy) ethane-N,N,N',N'-tetra acetic acid pentaacetoxymethyl ester (Indo 1/AM) (3 μg/ml), incubated at 37˚C for 30 min and analyzed by flow cytometry (17). The % of cells being stained by Indo 1/AM was calculated.

The effect of morin on mitochondrial membrane potential ($\Delta\Psi_m$) in HL-60 cells examined by flow cytometry. Approximately $5x10^5$ cells/well of HL-60 cells with morin (0, 100, 200, 300, 400 and 500 μM) were incubated in a 12-well plate for 24 h to detect the changes of $\Delta\Psi_m$. The cells were harvested and washed twice, re-suspended in 500 μl of 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) (4 mol/L), incubated at 37˚C for 30 min and analyzed by flow cytometry (15, 16). The % of cells being stained by $DiOC_6$ was calculated.

The effect of morin on the expressions of Wee1, cyclin A, cyclin B1, Cdc25c, CHK2, p21, caspase-9, caspase-3, Bcl-XL and Bax of HL-60 cells. The total proteins were collected from HL-60 cells after treatment with various concentrations of morin (0, 100, 200, 300, 400 and 500 μM) for 48 hours before the Wee1, cyclin A, cyclin B1, Cdc25c, CHK2, p21, caspase-9, caspase-3, Bcl-XL and Bax levels were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as previously described (13).

Statistical analysis. The Student's *t*-test was used to analyze the differences between the morin treated and control groups.

Results

Morin induced morphological changes and decreased the percentage of viable HL-60 cells. The results demonstrated that morin induced morphological changes and this effect was time-dependent (Figure 1A). Also, morin decreased the percentage of viable cells at a dose- and time-dependent manner (Figure 1B and C).

Morin induced cell cycle arrest and apoptosis in HL-60 cells. After HL-60 cells were treated with various concentrations of morin for various time periods, cell cycle and apoptosis (sub-G_1-phase) were examined and analyzed by flow cytometric methods. The representative profiles of cell cycle and apoptosis are given in Figure 2A. The data demonstrated that morin induced G_2/M arrest (Figure 2B) and apoptosis (Figure 2C). These effects were also time dependent.

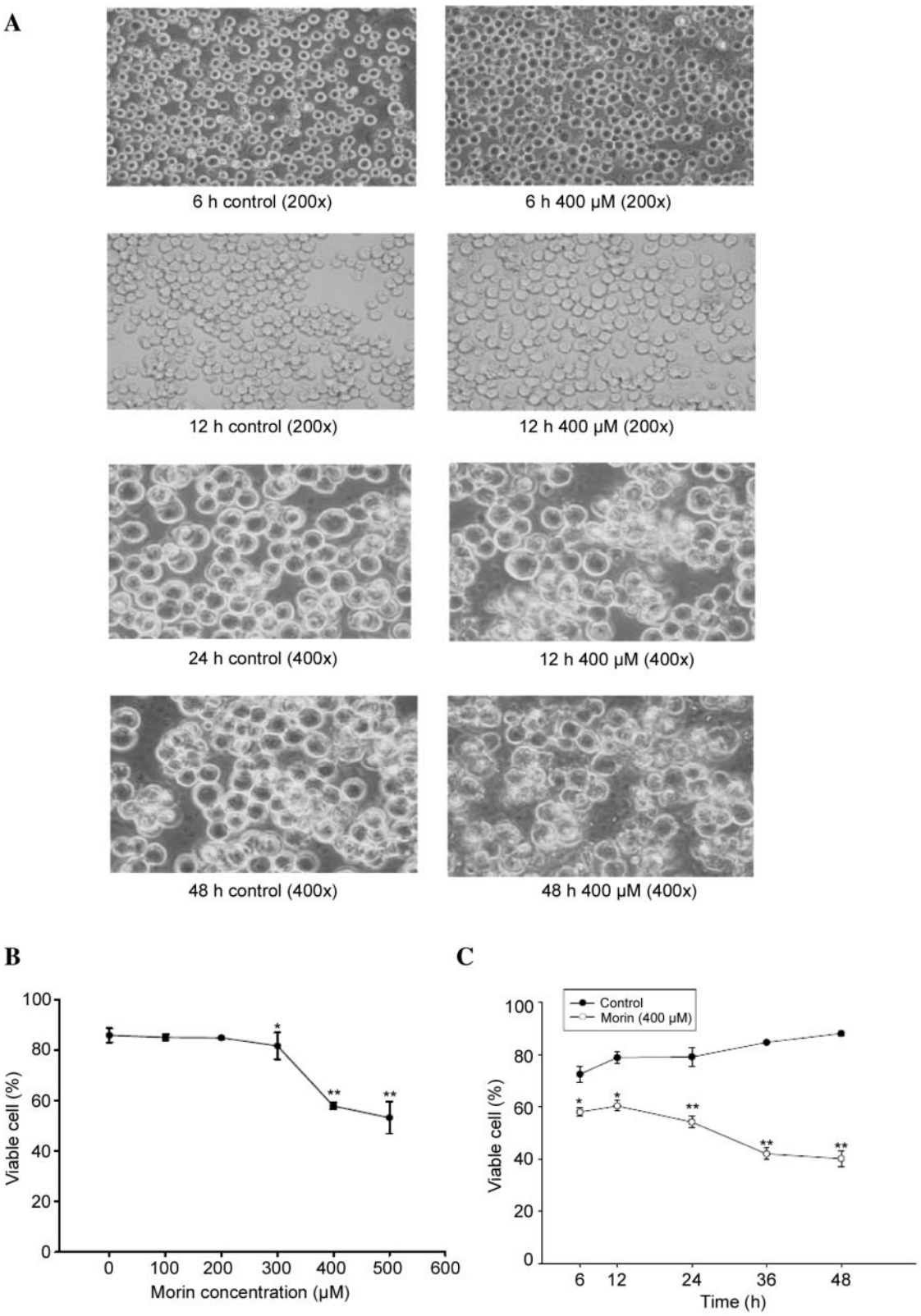


Figure 1. *Morphological changes and percentage of the viable HL-60 cells after morin treatment. The cells were photographed (A) and collected by centrifugation and viability (B and C) was determined by trypan blue exclusion and flow cytometry. Each point is mean±S.D. of three experiments. *Significant differences between morin-treated cells and control. *p<0.05.*

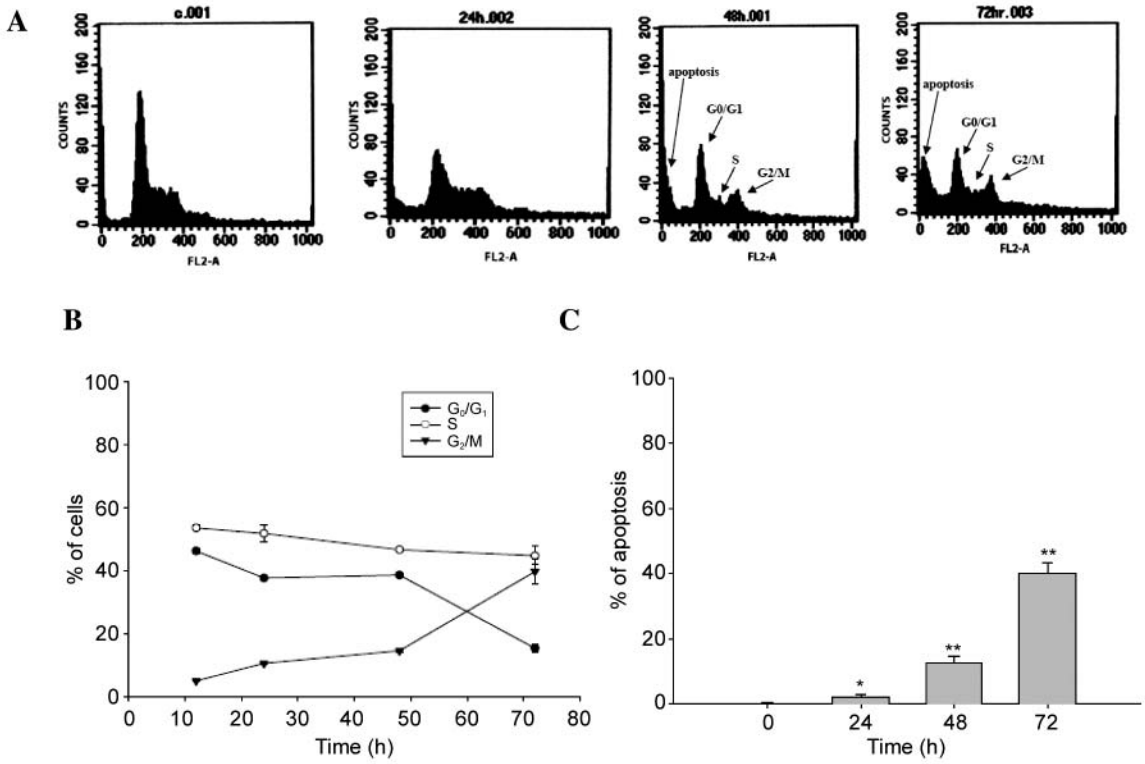


Figure 2. *Flow cytometric analysis of the effects of morin on the HL-60 cell cycle and sub-G_1 group. A) Representative profiles of cell cycle; B) percent of cells in various phases and sub-G_1-phase; and C) percent of cells in apoptosis were analyzed by flow cytometry. Data represents mean±S.D. of three experiments. *Significant differences between morin-treated cells and control. *p<0.05.*

Morin induced apoptosis in HL-60 cells examined by DAPI staining. After HL-60 cells were treated with various concentrations of morin for 48 h, and stained with DAPI for apoptosis. Representative pictures are given in Figure 3. The results demonstrated that morin induced apoptosis is a dose-dependent manner.

Morin induced DNA fragmentation in HL-60 cells. After HL-60 cells were treated with various concentrations of morin for 48 hours, DNA fragmentation was analyzed by DNA gel electrophoresis. The representative picture of DNA fragmentation is given in Figure 4. The results demonstrated that morin induced apoptosis in a dose-dependent manner.

Morin promoted the caspase-3 activity in HL-60 cells. Representative profiles of caspase-3 activity are given in Figure 5A. The data demonstrated that morin promoted the activation of caspase-3 (Figure 5B) and these effects was dose-dependent.

Morin induced the production of reactive oxygen species in HL-60 cells. After HL-60 cells were treated with various concentrations of morin for various time periods, ROS productions was analyzed and quantified by flow cytometric methods. Representative profiles of ROS are given in Figure 6A. The data demonstrated that morin induced ROS production in a dose- (Figure 6B) and time-dependent manner cure (Figure 6C).

Morin induced the Ca^{2+} production in HL-60 cells. After HL-60 cells were treated with various concentrations of morin for 24 h, Ca^{2+} production was quantified by flow cytometric methods. Representative profiles given in Figure 7A indicate that increased dose of morin led to an increase in the Ca^{2+} production by the cells (Figure 7B).

Morin decreased the levels of mitochondria membrane potential ($\Delta\Psi_m$) in HL-60 cells. After HL-60 cells were treated with various concentrations of morin for 12 h, the levels of $\Delta\Psi_m$ were analyzed and quantified by flow cytometric methods. The representative profiles given in Figure 8A indicate that increased dose of morin led to a decrease in the levels of $\Delta\Psi_m$ (Figure 8B) (% of cells were stained by $DiOC_6$).

The effects of morin on the expressions of Wee1, cyclin A, cyclin B1, Cdc25c, CHK2, p21, caspase-9, caspase-3, Bcl-XL and Bax in HL-60 cells. The results after treatment of HL-60 cells with various concentrations of morin and examination by Western

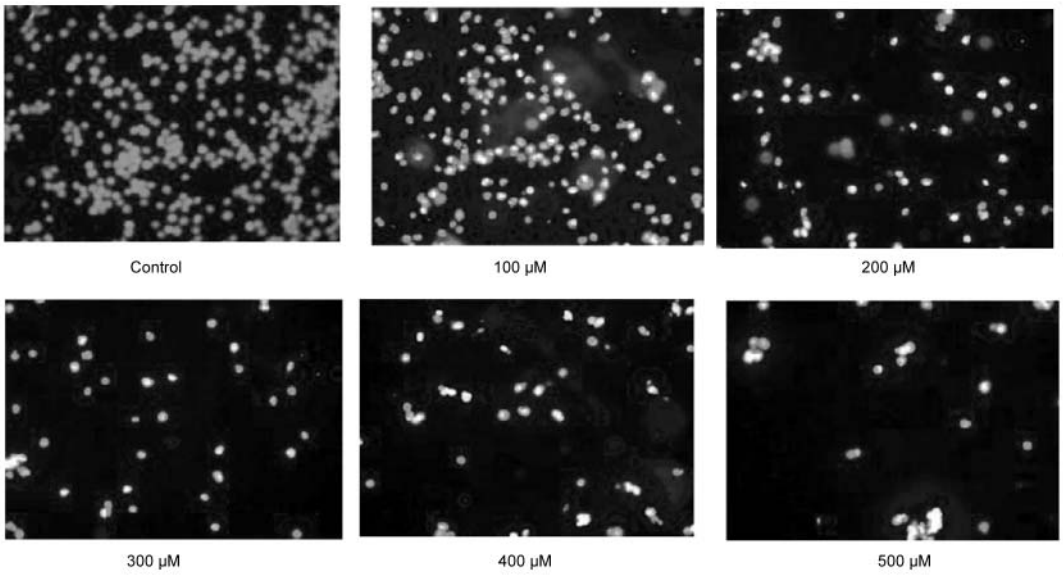


Figure 3. *The induction of apoptosis in HL-60 cells after treatment with morin was examined by DAPI staining.*

blotting [Figure 9 (Panel A: Wee1; Panel B: cyclin A; Panel C: cyclin B1; Panel D: Cdc25c; Panel E: CHK2; Panel F: p21; Panel G: caspase-9; Panel H: caspase-3; Panel I: Bcl-xL and Panel J: Bax)] indicate that the levels of cyclins A and B1, cdc25c were decreased, while Wee1 was overexpressed. This may have led to G_2/M arrest. Morin increased the expressions of p21, Bax, caspase-3 and -9 and decreased the expression of Bcl-xL. This may have led to apoptosis in HL-60 cells.

Discussion

Although the anticancer action of morin is still unclear, our results clearly demonstrated that morin induced G_2/M-phase arrest and apoptosis in human leukemia HL-60 cells. This is in agreement with other reports which demonstrated that morin induced apoptosis in human prostate cancer LNCaP cells (9). Our results also demonstrated that morin decreased the percentage of viable cells, promoted ROS and Ca^{2+} productions, decreased mitochondria membrane potential, promoted caspase-3 and -9 activation and led to apoptosis (DNA fragmentation and sub-G_1-phase of cell cycle). These effects were dose- and time-dependent. Our data also showed that morin induced apoptosis through a mitochondria-dependent pathway.

ROS production in HL-60 cells after treatment with morin occurred relatively quickly. ROS may lead to increased production of p21 protein. But after 24 hours ROS was decreased. Although it has been reported that low levels of ROS enhanced cell proliferation (18, 19), the production of ROS may contribute to tumor progression (20). An interesting point is that antioxidants scavenged intracellular ROS which led to suppression of proliferation of transformed cells (21)

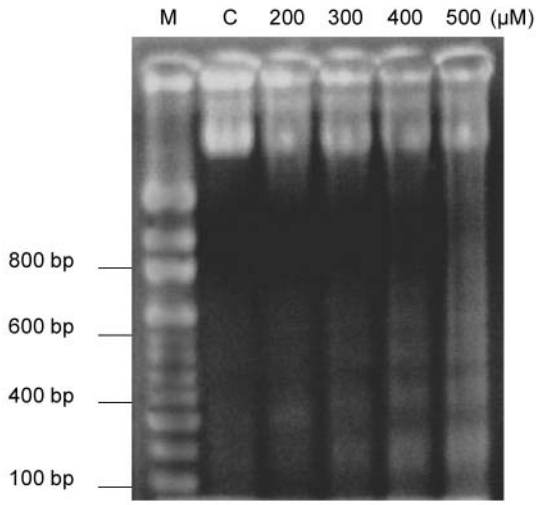


Figure 4. *Gel electrophoresis for determining DNA fragmentation in human HL-60 cells after treatment with morin.*

and colony formation (22). Our results have also shown that morin-induced apoptosis occurred after HL-60 cells were treated with morin for 24 hours. But the decrease of percentage of viable cells had already been shown after 6 hours. Apparently the decreased percentage of viable cells is not directly associated with apoptosis. This is in agreement with other results which have demonstrated that growth inhibition by morin does not seem to be associated with induction of apoptosis in oral cancer cell cultures (11).

It is well-documented that cell cycle checkpoints (associated enzyme complexes) play an important role in ensuring the proper execution of cell cycle events. Enzymes, such as CDK,

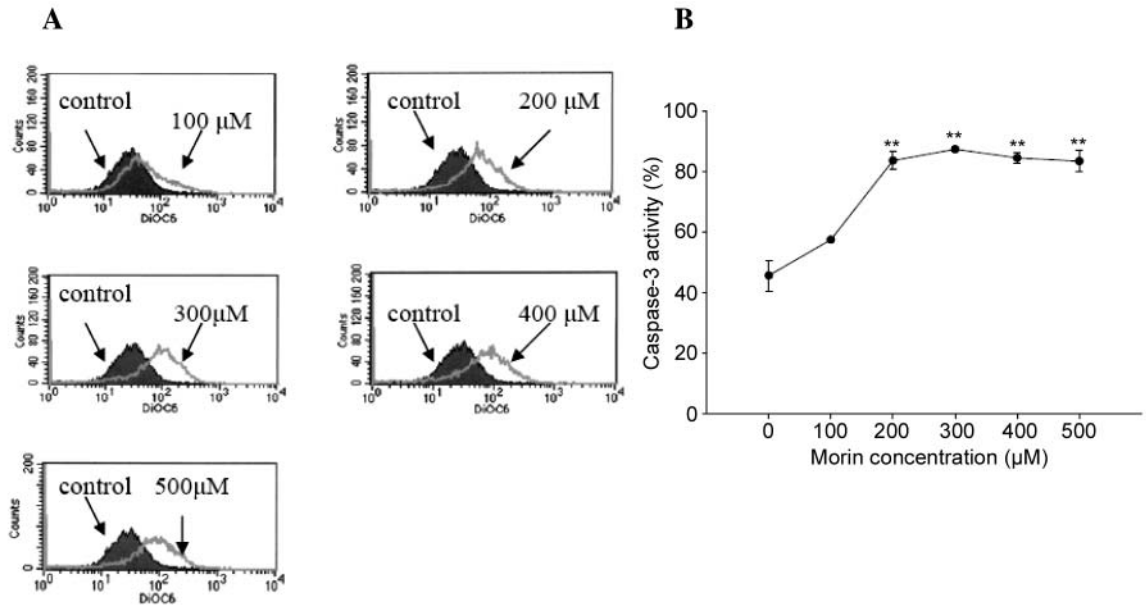


Figure 5. *Flow cytometric analysis of the effects of morin on caspase-3 activity in HL-60 cells. A) Representative profiles of caspase-3 activity; and B) percent of caspase-3 activity. Data represent mean±S.D. of three experiments. *Significant differences between morin-treated cells and control. *p<0.05.*

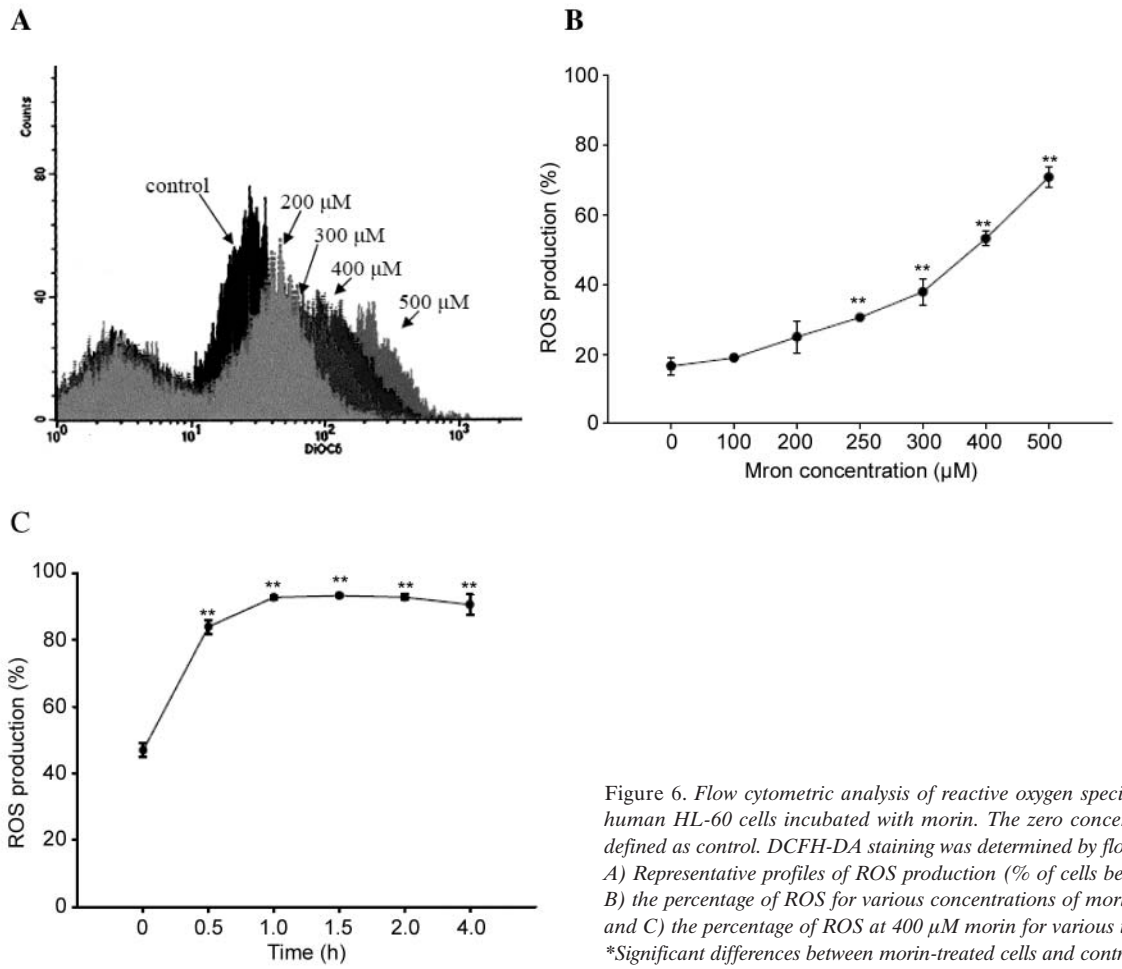


Figure 6. *Flow cytometric analysis of reactive oxygen species (ROS) in human HL-60 cells incubated with morin. The zero concentration was defined as control. DCFH-DA staining was determined by flow cytometry. A) Representative profiles of ROS production (% of cells being stained); B) the percentage of ROS for various concentrations of morin treatment; and C) the percentage of ROS at 400 µM morin for various time periods. *Significant differences between morin-treated cells and control. *p<0.05.*

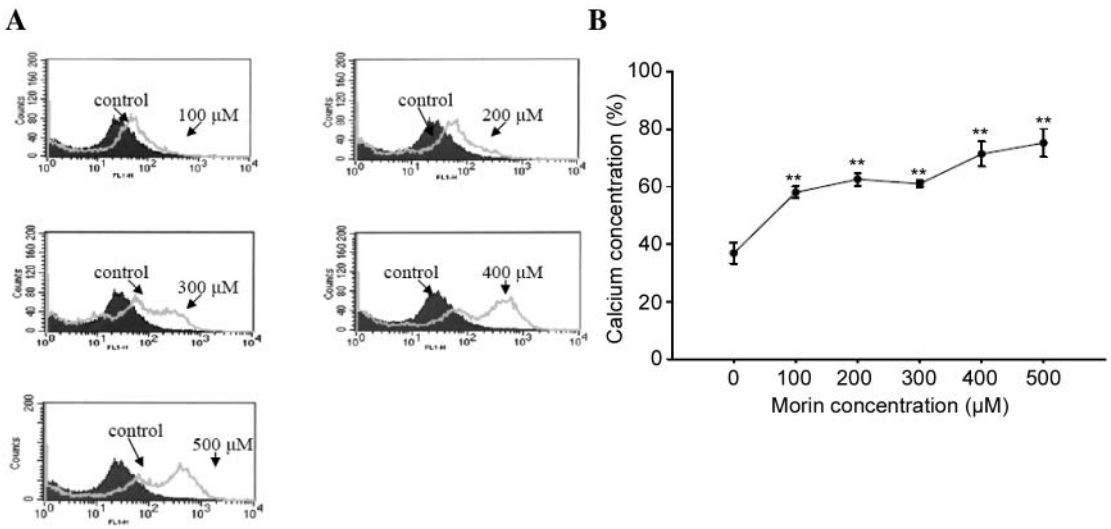


Figure 7. *Flow cytometric analysis of Ca^{2+} concentration in human HL-60 cells incubated with morin for 24 hours. Zero concentration was defined as control. Indo-1/AM staining was determined by flow cytometry. A) Representative profiles of Ca^{2+}; B) the percentage of Ca^{2+} concentrations. *Significant differences between morin-treated cells and control. *$p<0.05$.*

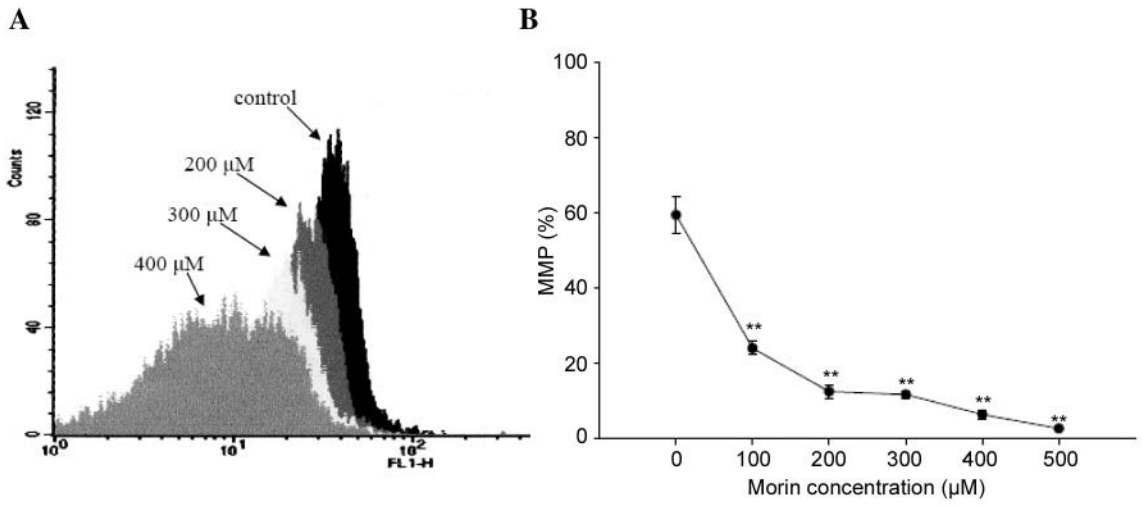


Figure 8. *Flow cytometric analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in human HL-60 cells incubated with morin. Zero concentration of morin was defined as control. $DiOC_6$ staining was determined by flow cytometry. A) Representative profiles of $\Delta\Psi_m$; B) the percentage of $\Delta\Psi_m$ for various concentrations of morin. *Significant differences between morin-treated cells and control. *$p<0.05$.*

and the cyclins are the major molecular players in cell cycle progression. The inhibition of CDK activity leads to cell cycle arrest, therefore the molecules which can inhibit CDKs may be anticancer agents specifically targeting the cell cycle. It has been reported that the CDK inhibitors (flavopiridol, UCN-01, olomoucine, rocosvitine, butyrolactone I, indirubin-5-sulfonate, and indirubin-3'-monoxime) may inhibit the growth of cancer cells by causing cell cycle arrest and apoptosis (23). Our results have also shown that morin induced G_2/M arrest in HL-60 cells in a dose-and time-dependent manner. Based on the results from Western blotting, it was demonstrated that morin inhibited the levels of cyclin A and B1, CDK1, Cdc25 c and promoted the expression of Wee1 and those observations were associated with the G_2/M arrest. The clinically used anticancer drugs, such as taxol, have been shown to induce G_2/M arrest and apoptosis. More importantly taxol also affected these cycle-associated proteins. When the CDKs are inhibited and the cell undergoes cell-cycle arrest, the cell may have the opportunity to repair its own damaged DNA before it resumes cell proliferation or it may trigger the apoptotic machinery and the cell will undergo apoptosis because it continues to cycle with its damaged DNA. Western blotting

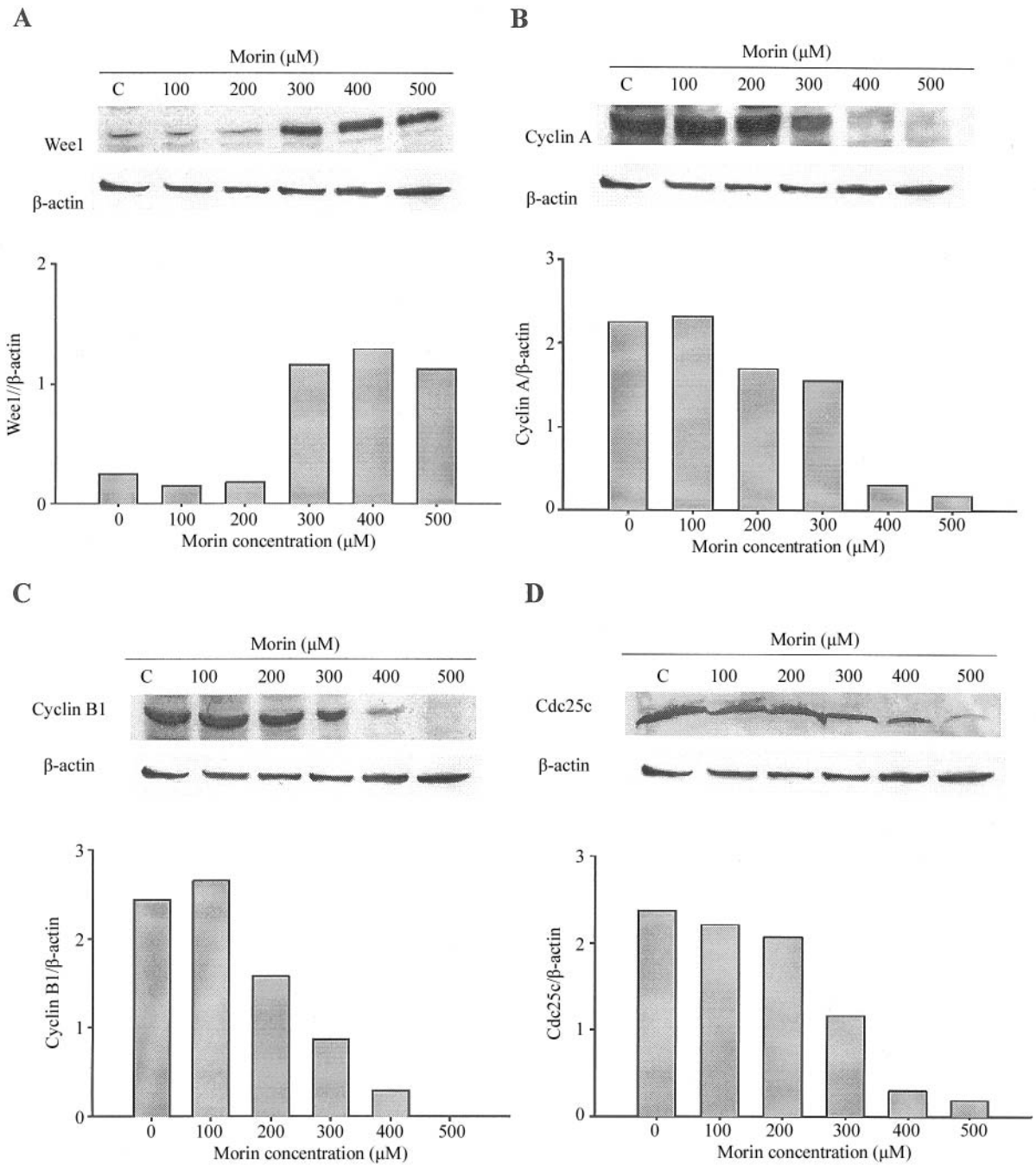
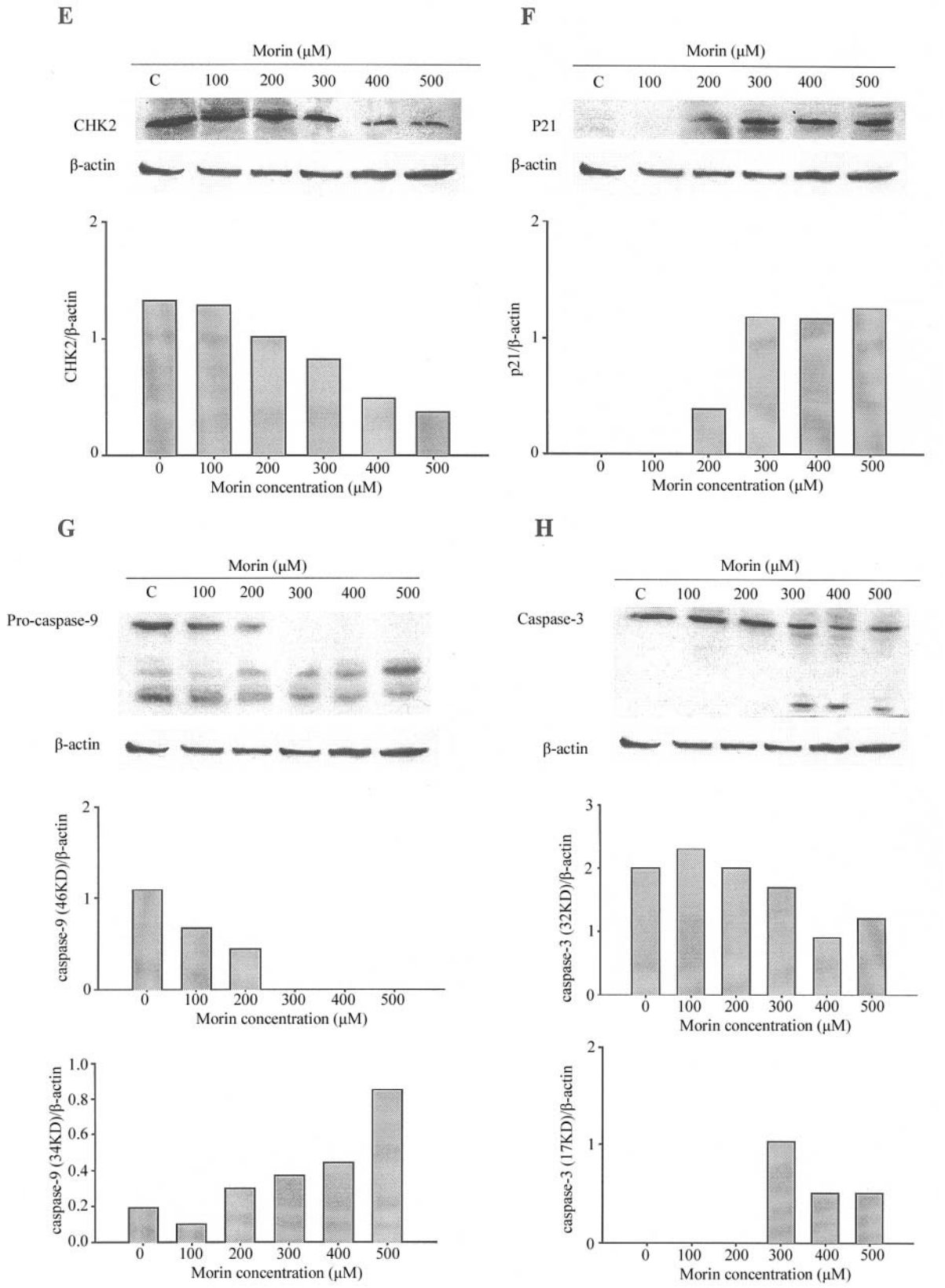


Figure 9. *continued*

also showed that morin promoted Bax expresssion (which is a pro-apoptotic protein) and increased cytochrome *c* release due to the decreased $\Delta\Psi_m$ and promoted the activation of caspase-3 and -9.

It is well-known that mitochondria play an important key role in the regulation of apoptosis (24, 25). Apoptosis can be divided into mitochondria-dependent and -independent pathways. However, mitochondrial dysfunctions, including the loss of mitochondrial membrane potential, permeability transition and release of cytochrome *c* from the mitochondria into the cytosol, are associated with apoptosis (26). Thus, a mitochondrial damage-dependent pathway might be involved in the morin-induced apoptosis in HL-60 cells (Figure 10). Other investigators have also demonstrated that morin affected



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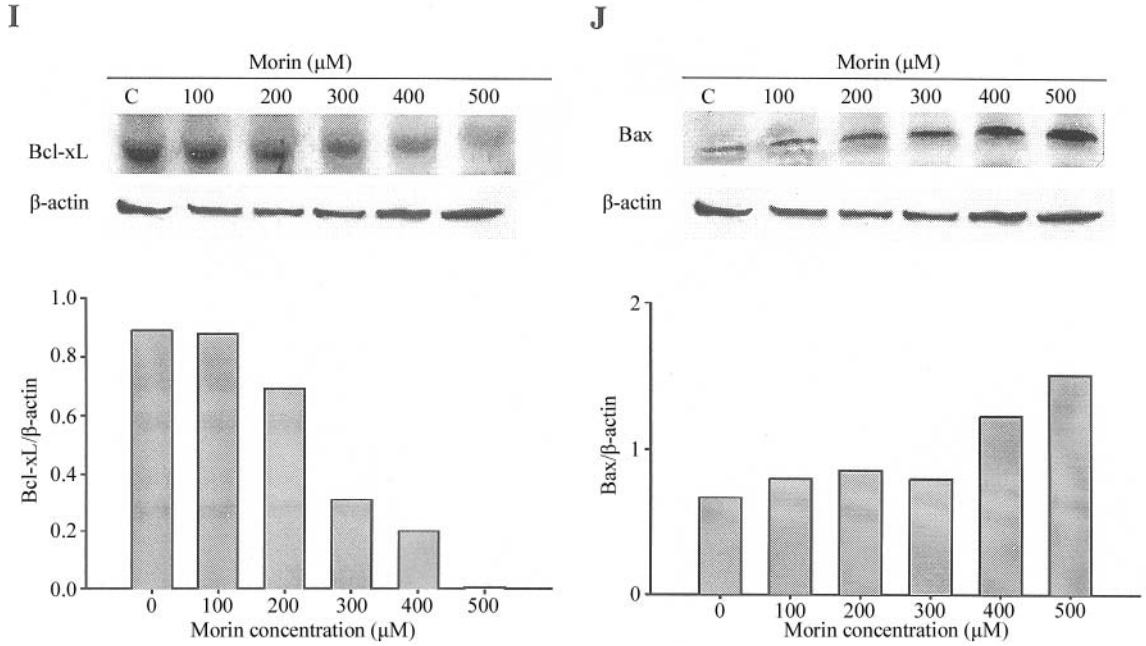

Figure 9. *Representative Western blot showing changes in the levels of cell cycle and apoptosis associated proteins in HL-60 cells after treatment with morin. The HL-60 cells (5x10^6/ml) were treated with 0, 100, 200, 300, 400 and 500 μM morin for 24 h then cytosolic fraction and total protein were prepared and determined, as described in "Materials and Methods". A) Wee1, B) cyclin A, C) cyclin B1, D) Cdc25c, E) CHK2, F) p21, G) caspase-9, H) caspase-3, I) Bcl-xLæ, and J) Bax were estimated by Western blotting, as described in the "Materials and Methods".*

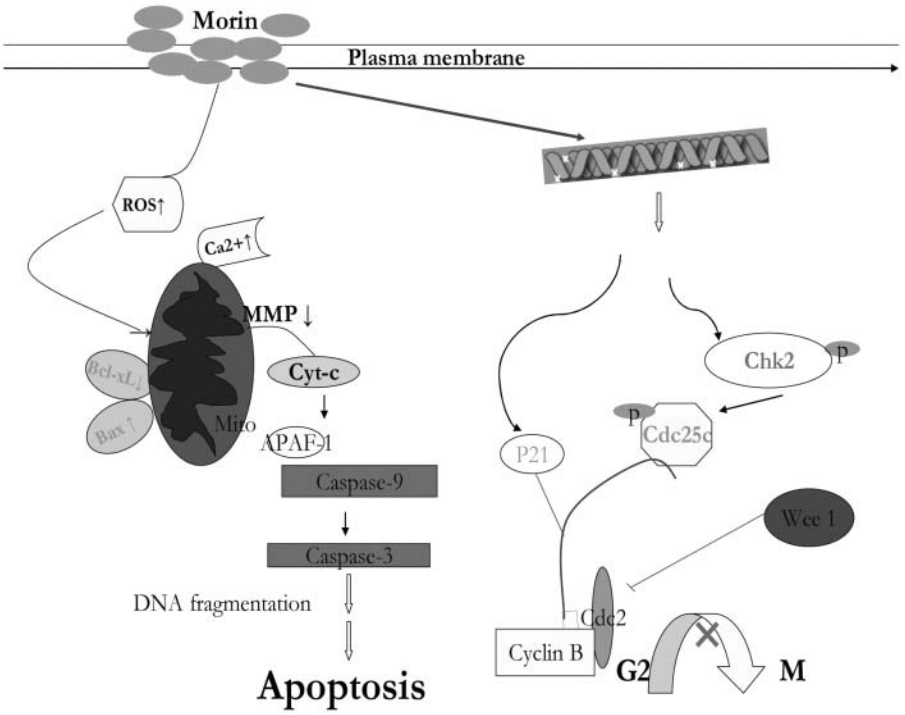

Figure 10. *Proposed model of morin mechanism of action for G_2/M arrest and apoptosis in HL-60 cells. Morin enhanced Wee1and p21 expression and decreased cyclin A, cyclin Bs, Cdc25c which led to G_2/M arrest. Morin increased the production of Bax, caspase-3 and -9 and decreased Bcl-xL which caused apoptosis in HL-60 cells.*

the main kinase signaling pathways and inhibited growth of oral cells, while the activation of the JNK and p38 stress kinase pathways [implicated in growth arrest of cells in response to stress signals of various kinds (27)], and inhibition of the PK-B/AKT pathway [considered to be mainly involved with regulating cell survival (28)] seem to be involved. It appears that morin induced cell cycle arrest and inhibited cell growth.

It is well known that caspases play a critical role in the initiation of apoptosis. According to the substrate specificities of caspases, they can be grouped into "apoptotic initiators", such as caspase-8, and "apoptotic effectors", such as caspase-3 (29). Our data demonstrated that morin-induced apoptosis was observed from 24 h up to 72 h (time-dependent manner). Morin promoted the activation of caspase-3, Bax, caspase-3 and -9 expression and cytochrome *c* release from mitochondria and decreased Bcl-2 expression in HL-60 cells. These results demonstrated that morin-induced apoptosis might involve a mitochondria-dependent pathway and caspase-3-mediated mechanism (Figure 10).

Acknowledgements

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References

1 Droge W: Free radicals in the physiological control of cell function. Physiol Rev *82*: 47-95, 2002.
2 Cerutti PA: Oxy-radicals and cancer. Lancet *344*: 862-863, 1994.
3 Wiseman H and Halliwell B: Damage to DNA by reactive oxygen and nitrogen species: Role in inflammatory disease and progression to cancer. Biochem J *313(Pt 1)*: 17-29, 1996.
4 Savill J and Haslett C: Granulocyte clearance by apoptosis in the resolution of inflammation. Semin Cell Biol *6*: 385-393, 1995.
5 Paschka AG, Butler R and Young CY: Induction of apoptosis in prostate cancer cell lines by the green tea component, (–)-epigallocatechin-3-gallate. Cancer Lett *130*: 1-7, 1998.
6 Smets LA: Programmed cell death (apoptosis) and response to anti-cancer drugs. Anticancer Drugs *5*: 3-9, 1994.
7 Windholz M: The Merck Index, eleventh ed. Merck & Co., Inc. pp. 986-987, 1989.
8 Park SY, Park SH, Lee IS and Kong JY: Establishment of a high-throughput screening system for caspase-3 inhibitors. Arch Pharm Res *23*: 246-251, 2000.
9 Romero I, Paez A, Ferruelo A, Lujan M and Berenguer A: Polyphenols in red wine inhibit the proliferation and induce apoptosis of LNCaP cells. BJU Int *89*: 950-954, 2002.
10 Alexandrakis M, Letourneau R, Kempuraj D, Kandere-Grzybowska K, Huang M, Christodoulou S, Boucher W, Seretakis D and Theoharides TC: Flavones inhibit proliferation and increase mediator content in human leukemic mast cells (HMC-1). Eur J Haematol *71*: 448-454, 2003.
11 Brown J, O'Prey J and Harrison PR: Enhanced sensitivity of human oral tumours to the flavonol, morin, during cancer progression: Involvement of the akt and stress kinase pathways. Carcinogenesis *24*: 171-177, 2003.
12 Hsiang CY, Wu SL and Ho TY: Morin inhibits 12-O-tetradecanoylphorbol-13-acetate-induced hepatocellular transformation *via* activator protein 1 signaling pathway and cell cycle progression. Biochem Pharmacol *69*: 1603-1611, 2005.
13 Li TM, Chen GW, Su CC, Lin JG, Yeh CC, Cheng KC and Chung JG: Ellagic acid induced p53/p21 expression, G_1 arrest and apoptosis in human bladder cancer T24 cells. Anticancer Res *25*: 971-979, 2005.
14 Packard BZ, Toptygin DD, Komoriya A and Brand L: Profluorescent protease substrates: Intramolecular dimers described by the exciton model. Proc Natl Acad Sci USA *93*: 11640-11645, 1996.
15 Lu HF, Sue CC, Yu CS, Chen SC, Chen GW and Chung JG: Diallyl disulfide (DADS) induced apoptosis undergo caspase-3 activity in human bladder cancer T24 cells. Food Chem Toxicol *42*: 1543-1552, 2004.
16 Lin SS, Chung JG, Lin JP, Chuang JY, Chang WC, Wu JY and Tyan YS: Berberine inhibits arylamine N-acetyltransferase activity and gene expression in mouse leukemia L 1210 cells. Phytomedicine *12*: 351-358, 2005.
17 Park EK, Kwon KB, Park KI, Park BH and Jhee EC: Role of Ca(2+) in diallyl disulfide-induced apoptotic cell death of HCT-15 cells. Exp Mol Med *34*: 250-257, 2002.
18 Kamata H and Hirata H: Redox regulation of cellular signalling. Cell Signal *11*: 1-14, 1999.
19 Benhar M, Engelberg D and Levitzki A: ROS, stress-activated kinases and stress signaling in cancer. EMBO Rep *3*: 420-425, 2002.
20 Szatrowski TP and Nathan CF: Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res *51*: 794-798,1991.
21 Burdon RH: Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic Biol Med *18*: 775-794, 1995.
22 Jurgensmeier JM, Panse J, Schafer R and Bauer G: Reactive oxygen species as mediators of the transformed phenotype. Int J Cancer *70*: 587-589, 1997.
23 Buolamwini JK: Cell cycle molecular targets in novel anticancer drug discovery. Curr Pharm Des 6: 379-392, 2000.
24 Desagher S and Martinou JC: Mitochondria as the central control point of apoptosis. Trends Cell Biol *10*: 369-377, 2000.
25 Gottlieb RA: Mitochondria: Execution central. FEBS Lett *482*: 6-12, 2000.
26 Xia Z, Lundgren B, Bergstrand A, DePierre JW and Nassberger L: Changes in the generation of reactive oxygen species and in mitochondrial membrane potential during apoptosis induced by the antidepressants imipramine, clomipramine, and citalopram and the effects on these changes by bcl-2 and bcl-X(L). Biochem Pharmacol *57*: 1199-1208, 1999.
27 Kyriakis JM and Avruch J: Sounding the alarm: Protein kinase cascades activated by stress and inflammation. J Biol Chem *271*: 24313-24316, 1996.
28 Franke TF, Kaplan DR and Cantley LC: PI3K: Downstream AKTion blocks apoptosis. Cell *88*: 435-437, 1997.
29 Kaufmann SH and Hengartner MO: Programmed cell death: Alive and well in the new millennium. Trends Cell Biol *11*: 526-534, 2001.

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