ANTICANCER RESEARCH *24*: 1769-1778 (2004)

Selective Induction of G2/M Arrest and Apoptosis in HL-60 by a Potent Anticancer Agent, HMJ-38

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Abstract. *We previously reported that HMJ-38 was the most potent 2-phenyl-4-quinozolinone derivative in inhibiting tubulin polymerization and showed significant cytotoxicity against several human tumor cell lines. In this work, we studied its cytotoxic effect on HL-60 leukemia cells and the underlying mechanisms. We first investigated the effects of HMJ-38 on viability, cell cycle and induction of apoptosis in HL-60 and normal human peripheral blood mononuclear cells (PBMC). After 24-hour treatment with HMJ-38, a dose- and time-dependent decrease in the viability of HL-60 cells was observed and the approximate IC_{50} was 4.48* μM. *The cytotoxic effect of HMJ-38 on PBMC was less significant than that on HL-60 cells, either with 24 or 48 hours of treatment. Cell cycle analysis showed that HMJ-38 induced significant G2/M arrest and apoptosis in HL-60 cells. The HMJ-38-induced G2/M arrest occurred before the onset of apoptosis. Within 24 hours of treatment, HMJ-38 influenced the CDK/cyclin B activity by increasing Chk1, Wee1 and p21 and decreasing Cdc25C protein levels. The HMJ-38-induced apoptosis was further confirmed by morphological assessment and DNA fragmentation assay. Induction of apoptosis in HMJ-38-treated HL-60 cells was accompanied by an apparent increase of cytosolic cytochrome c, down-regulation of Bcl-2, up-regulation of Bax and cleavage of pro-caspase-9, -3 and poly(ADP)ribosylpolymerase (PARP). The results of the significant reduction of caspase activities and apoptosis by caspase inhibitors indicated that the HMJ-38-induced apoptosis was mainly mediated by activation of caspases-9 and -3. HMJ-38 also activated ERK in HL-60 cells. Pre-incubating cells with ERK inhibitors (U0126 and PD98059) attenuated the HMJ-38-induced ERK activation and apoptosis. Nevertheless, cells remained arrested in G2/M. These results suggest that HMJ-38 is a potent anticancer drug and it shows a remarkable action on cell cycle before commitment for apoptosis is reached.*

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Key Words: 2-(3'-methoxyphenyl)-6-pyrrolidinyl-4-quinazolinone, HL-60, PBMC, apoptosis, cell cycle, G2/M arrest, ERK.

The clinical applicability of an anticancer drug is defined by both its anticancer potency and its therapeutic index between cancerous and normal cells. A new drug that is not detrimental to normal cells and exerts its cytotoxic effects only on cancer cells would be the first choice for providing high chemotherapeutic effectiveness as well as limiting side-effects such as decrease of human immunity (1). Recently, we have designed and synthesized a series 2-phenyl-4-quinazolinone derivatives as novel antimitotic agents, among which HMJ-38 (2-(3'-Methoxyphenyl)-6-pyrrolidinyl -4-quinazolinone, as shown in Figure 1) is the most potent. HMJ-38 significantly inhibited tubulin polymerization and showed notable cytotoxic effect on 10 human tumor cell lines (1A9, HCT-8, A-549, U-87-MG, HOS, KB, KB-VIN, PC3, MCF-7 and SKMEL-2) (2). However, neither the cytotoxic effects of HMJ-38 on leukemia cells and normal peripheral blood mononuclear cells (PBMC), nor the molecular mechanisms underlying its anti-cancer activity have been revealed.

The progression of the cell cycle has been intensively investigated (3). Cyclin-dependent protein kinase 1 (CDK1) along with cyclin B plays pivotal roles in the regulation of the cell cycle in G2/M-phase (4-6). In addition to the CDK1/cyclin B complex, progression from G2- to the M-phase can be affected by the phosphorylation status of CDK1. Cdc25C phosphatase accelerates mitosis entry by dephosphorylation of CDK1 at Thr-14 and Tyr-15 (7). On the other hand, Wee l kinase delays mitosis entry by phosphorylation of CDK1 at Tyr-15 (8). Chk1 functions as an essential kinase in the G2/M damage checkpoint by phosphorylating Cdc25C in response to DNA-damaging and

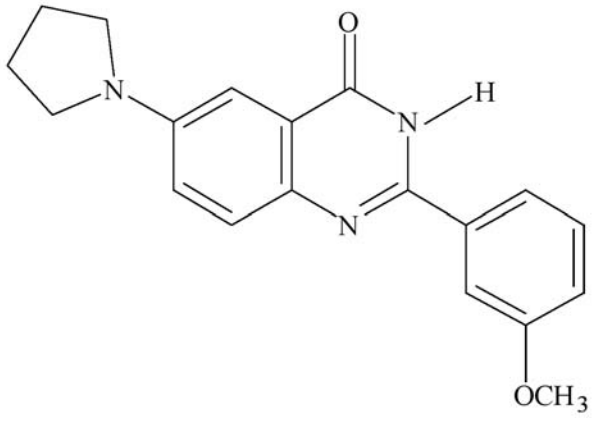

Figure 1. *Structure of 2-(3'-methoxyphenyl)-6-pyrrolidinyl-4-quinazolinone (HMJ-38).*

antimitotic agents, thereby blocking mitosis (9). In the meanwhile, the activity of CDK1/cyclin B complexes is blocked by $p21^{waf/cip1}$ protein (10). Several agents affecting microtubule assembly or disassembly have been shown to interfere with the progression of the cell cycle, and some of them show significant clinical anticancer effects. When exposed to microtubule-binding agents (such as vinca alkaloids and taxol), cells exhibited G2/M-phase arrest and changes involved in CDK1/cyclin B activity (11, 12).

In addition to cell cycle arrest, regulation and/or manipulation of apoptosis in tumor cells are prominent approaches for cancer therapy (13). Several anti-cancer agents can induce apoptosis (14). Substantial evidence showed that the induction of apoptosis is due to damage to the cell cycle or to DNA integrity. Cells undergoing apoptosis have observable morphology changes expressed as nuclear condensation, DNA fragmentation and compact packaging of the cellular debris into apoptotic bodies (15). During the process, sequential cleavage and activation of caspases is a requisite step of the apoptotic cascade (16). The initiator caspases include either caspase-8 in Fas or TNF-induced apoptosis, or caspase-9 whose activation is triggered by cytochrome c released from mitochondria in response to various stimuli. They can directly or indirectly activate downstream effectors caspase-3, -6 and -7. The activated caspase-3, -6 and -7 are supposed to cleave death substrates, such as lamins, poly(ADP-ribose) polymerase (PARP), *etc*. (17). The complicated apoptotic pathways are delicately modulated by pro-apoptotic and anti-apoptotic protein families such as the Bcl-2 family. The apoptotic regulator, Bcl-2, inhibits apoptosis but Bax promotes apoptosis by blocking Bcl-2 activity. The protein ratio of Bcl-2 and Bax has been recognized as a key factor in the regulation of the mitochondria-mediated apoptotic process (18). Besides, intracellular signaling molecules such as PKA, PKB, PKC, p38, JNK and ERK have also been shown to be involved in apoptosis control (19).

The mechanism by which HMJ-38 exerts cytotoxicity remains largely unknown. In the present study, we evaluated the cytotoxic effects of HMJ-38 on leukemia and normal human cells. The molecular mechanisms of its anti-cancer effects were investigated on human HL-60 leukemia cells. Here we report how HMJ-38 interferes with cell cycle progression and induced apoptosis.

Materials and Methods

Chemicals and reagents. Propidium iodide (PI), RNase A, Proteinase K and PHA (Sigma, MO, USA) were dissolved in H_2O. HMJ-38 (Figure 1), general caspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), caspase-9 inhibitor z-Leu-Glu-His-Asp-fluoromethyl ketone (z-LEHD-fmk) (R&D Systems, MN, USA), U0126 and PD98059 (Promega Corporation, WI, USA) were dissolved in dimethylsulfoxide (DMSO) (Sigma) and diluted in tissue culture medium before use.

Cell culture and HMJ-38 treatment. The human HL-60 leukemia cells were obtained from the Culture Collection and Research Center (CCRC) in Taiwan, originally from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 (GIBCO BRL, Life Technologies, MD, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone, UT, USA) at 37˚C, 5% CO_2 in a humidified incubator. Before HMJ-38 treatments, cells were suspended at a final density of $2.5x10^5$ cell/ml and seeded in 24-well plates (1ml/well) or 25T flasks (Falcon, CA, USA). The PBMC were isolated from heparinized whole blood of healthy volunteers by Ficoll-Paque Plus® (Amersham-Pharmacia Biotech AB, Uppsala, Sweden). After washing with PBS, the cells were suspended in 10% FCS/ RPMI-1640. PBMC ($1x10^6$), either stimulated by 5 μg/ml phytohaemagglutinin (PHA) for 48 h or not, were treated with HMJ-38 for 24-72 h (20).

Assessment for cell viability. Cell viability was determined by the PI exclusion method (21). At the indicated time course of HMJ-38 treatment, cells were collected, resuspended in PBS containing 4 μg/ml PI and then analyzed by flow cytometry (FACS Calibur™, Becton Dickinson, NJ, USA). All experiments were performed in triplicate. The percentage of cell viability was calculated as a ratio between drug-treated cells and 0.1% DMSO vehicle-control cells.

Analysis for DNA content. After the indicated treatments, cells were harvested and lysed in hypotonic PI solution (0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml PI). The cell cycle and apoptotic nuclei were determined by flow cytometry (FACS Calibur™, Becton Dickinson) (22).

Assay for DNA fragmentation. Cells were collected and lysed in lysis buffer (20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.2% Triton X-100). The cell lysate was treated with 0.1 μg/ml proteinase K at 50˚C overnight, followed by 50 μg/ml RNase A at 37˚C for 30 min. After precipitation, the DNA was subjected to electrophoresis in a 1.0% agarose gel. DNA fragmentation was visualized by ethidium bromide staining and examined in photographs taken under UV light (23).

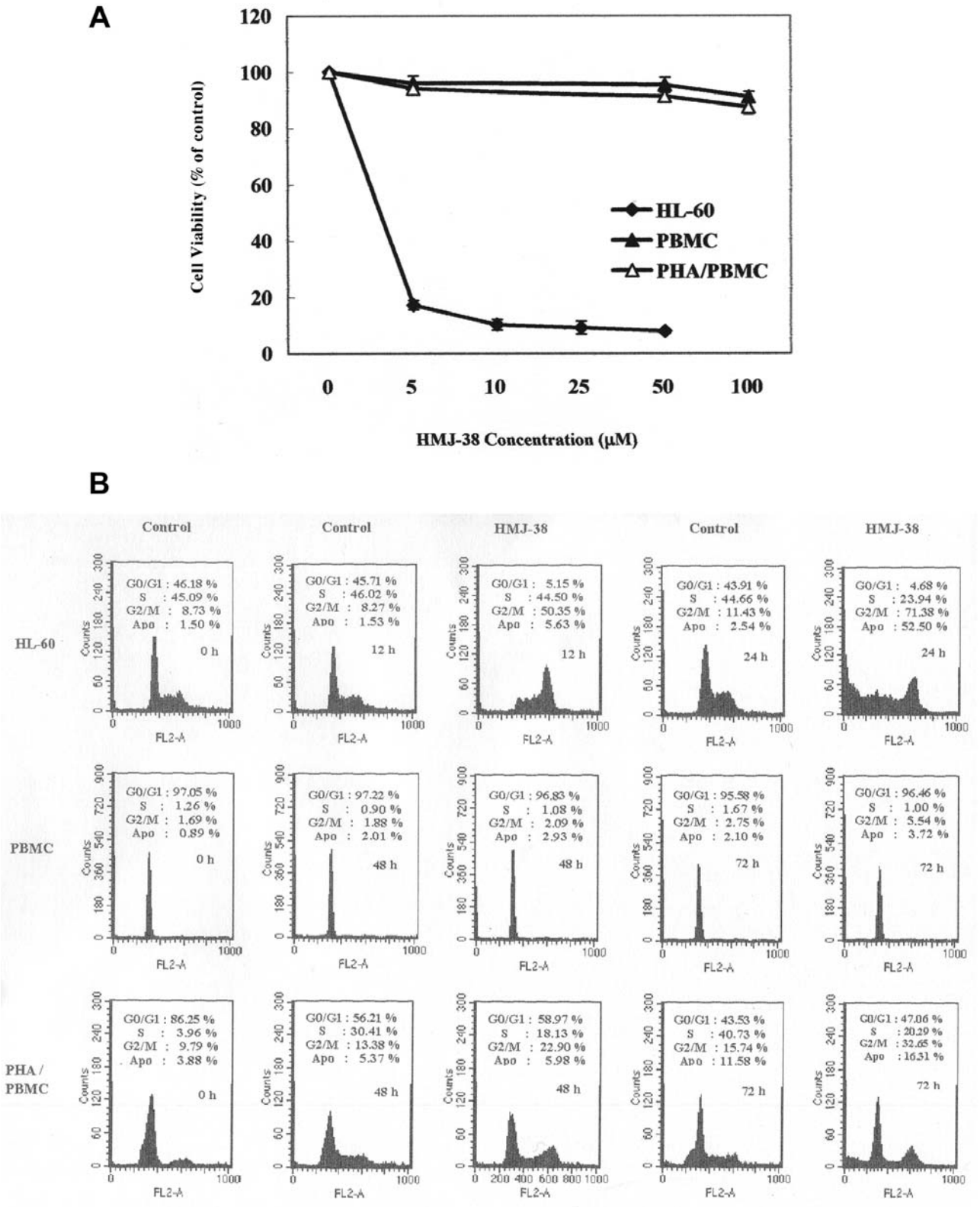

Figure 2. *Effects of HMJ-38 on cell viability and cell cycle progression. (A) The cell viability on HL-60, PBMC and PHA-stimulated PBMC after 24-hour treatment of HMJ-38. (B) DNA content in cells treated with 5 μM HMJ-38 at the time indicated. Viable cells and cell cycle progression were measured by PI exclusion and immediately analyzed by flow cytometry. *Apo indicated the Sub-G1 nuclei.*

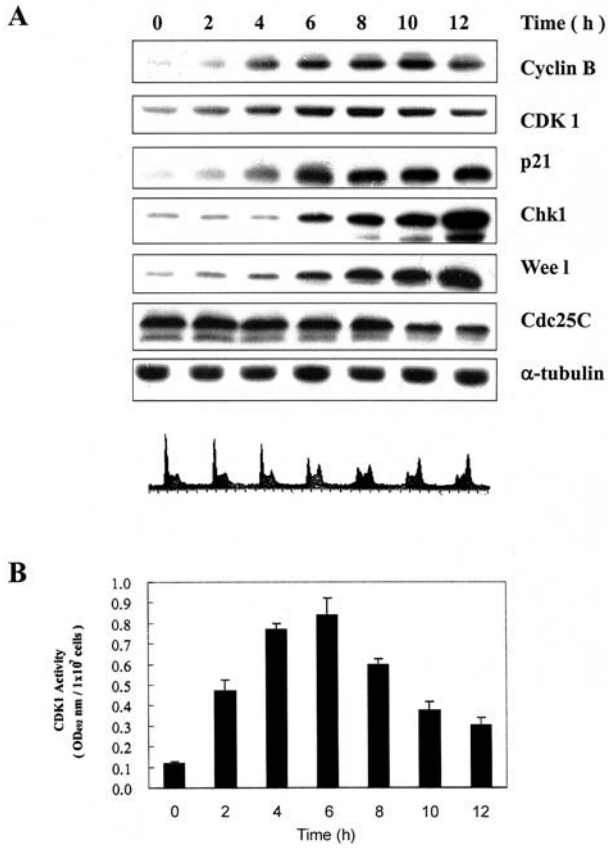

Figure 3. *Effects of HMJ-38 on the expression level changes of proteins involved in G2/M-phase and on cell cycle progression. (A) Western blot analysis of cyclin B, CDK1, p21, Chk1, Wee 1 and Cdc25C protein expression levels. (B) Kinase assay of CDK1 kinase activity. Total cellular extracts were subjected to SDS-PAGE and immunoblotted with antibodies specific to human p21, Cyclin B1, CDK1, Wee1, Chk1 and Cdc25C. CDK1 kinase activity was measured in cellular extracts for the ability to phosphorylate MV peptide, a CDK1 kinase specific substrate, according to the manual of the Medical & Biological Laboratory's CDK1 kinase assay kit.*

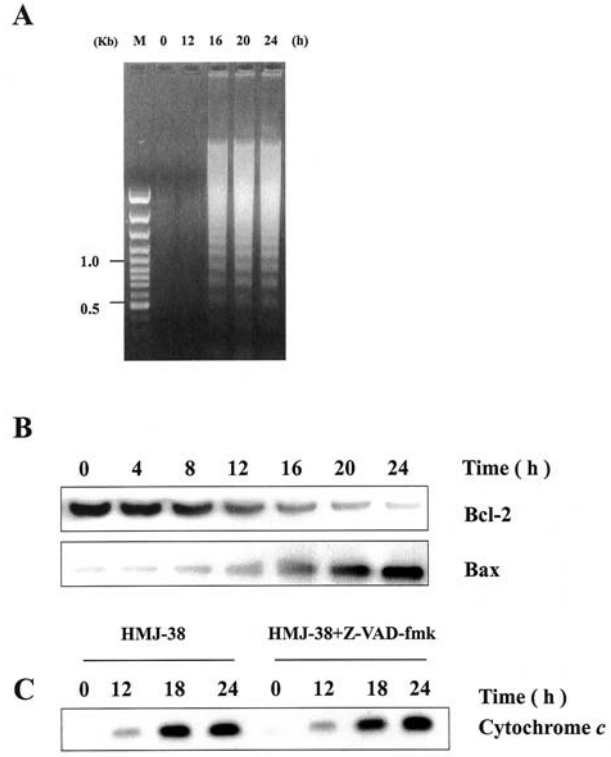

Figure 4. *Effects of HMJ-38-induced apoptosis in HL-60 cell on DNA fragmentation, changes in Bcl-2 and Bax protein levels and cytochrome c release in HL-60 cells. (A) DNA fragmentation. (B) Western blot analysis for Bcl-2 and Bax protein levels. (C) Western blotting analysis for cytosolic cytochrome c in 5 μM HMJ-38-treated cells, either in the presence or absence of pan-caspase inhibitor (Z-VAD-fmk) as indicated. For DNA fragmentation, DNA extracts were electrophoresed in 1.0% agarose gel. For Western blot analysis, total or cytosolic protein extracts were analyzed by immunoblotting with antibodies specific to human Bcl-2, Bax and cytochrome c.*

Western blot analysis. Cytosolic fraction and total protein were prepared and determined as previously described (24). Equal amounts of proteins (30 μg) were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). Blots were blocked in PBST buffer (0.05% Triton X-100 in PBS) containing 5% non-fat milk for 1 h, then the membrane was incubated overnight at 4˚C with specific primary antibodies: Cyclin B (NeoMarker, CA, USA), CDK1, Cdc25C, p21, cytochrome c, PARP (Pharmingen, CA, USA), Chk1, Ras (BD Transduction Laboratories, CA, USA), Wee1 (Santa Cruz Biotechnology, CA, USA), Bcl-2, Bax (Upstate Biotechnology, NY, USA), ERK protein, phospho-ERK protein, phospho-Elk, caspase-9, caspase-3 (R&D System) and α-tubulin (Amersham-Pharmacia Biotech AB). Subsequently, the membrane was washed with PBST buffer and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Santa Cruz Biotechnology, CA, USA). The specific protein was detected by using Western Blot Chemiluminescence Reagent *Plus* kits (NEN™ Life Science, MA, USA).

Assay for caspase-3, -8 and -9 activity. Cells were lysed in lysis buffer (50mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT). Cell lysates (50 μg protein) were incubated with caspase -3, -9 and -8 specific substrates (Ac-DEVD-pNA, Ac-LEHD-pNA and Ac-IETD-pNA; R&D System) for 1 h at 37˚C. The caspase activity was determined by measuring OD_{405} of the released pNA (25).

CDK1 kinase assay. CDK1 kinase activity was analyzed according to the protocol of Medical & Biological Laboratories CDK1 kinase assay kit (MBL, Medical & Biological Laboratories Co, Ltd, Japan) (26). In brief, the ability of the cell extract prepared from each treatment to phosphorylate its specific substrate, MV Peptide, was measured.

ERK enzymatic activity assay. ERK kinase activity was detected according to the protocol of the Cell Signaling Technology ERK kinase assay kit (Cell Signaling Technology, MA, USA) as

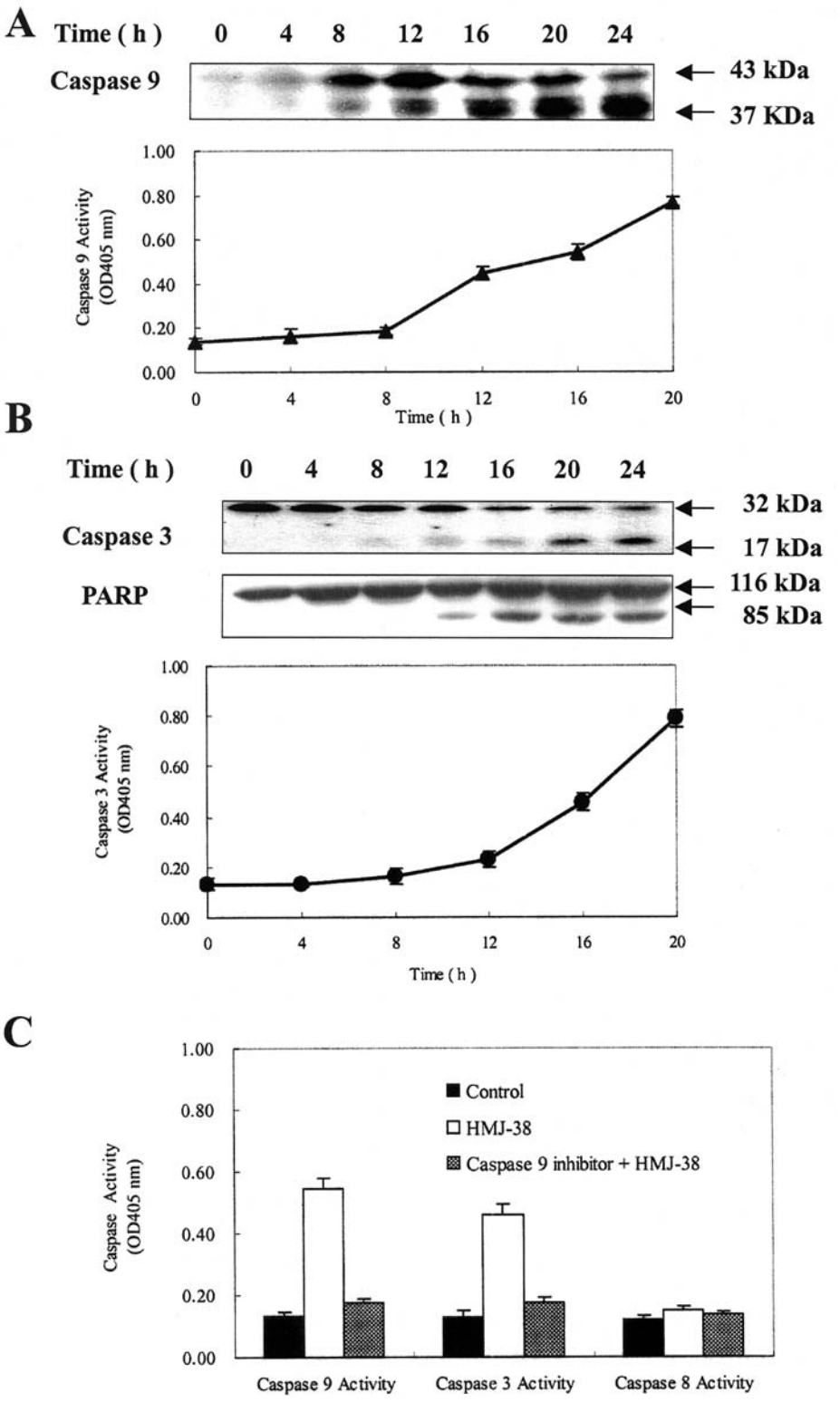

Figure 5. *HMJ-38 stimulated caspase activities and cleavage of PARP in HL-60 cell. Cells were treated with 5 μM HMJ-38 for the indicated time course. Total protein extracts were subjected to caspase activity assay and Western blotting analysis for caspase-9 and caspase-3 processing (A, B). The intact and cleaved products of caspases are indicated. (C) To determine the specific effect of caspase-9 inhibitor (z-LEHD-fmk) on 5 μM HMJ-38-treated cells, cells were co-incubated either with or without caspases-9 inhibitor. After 24 hours, aliquots of total cell extracts were incubated with caspases-3-, -9 and -8 specific substrates, respectively (Ac-DEVD-pNA, Ac-LEHD-pNA and Ac-IETD-pNA). The release of pNA was measured at 405 nm by a spectrophotometer.*

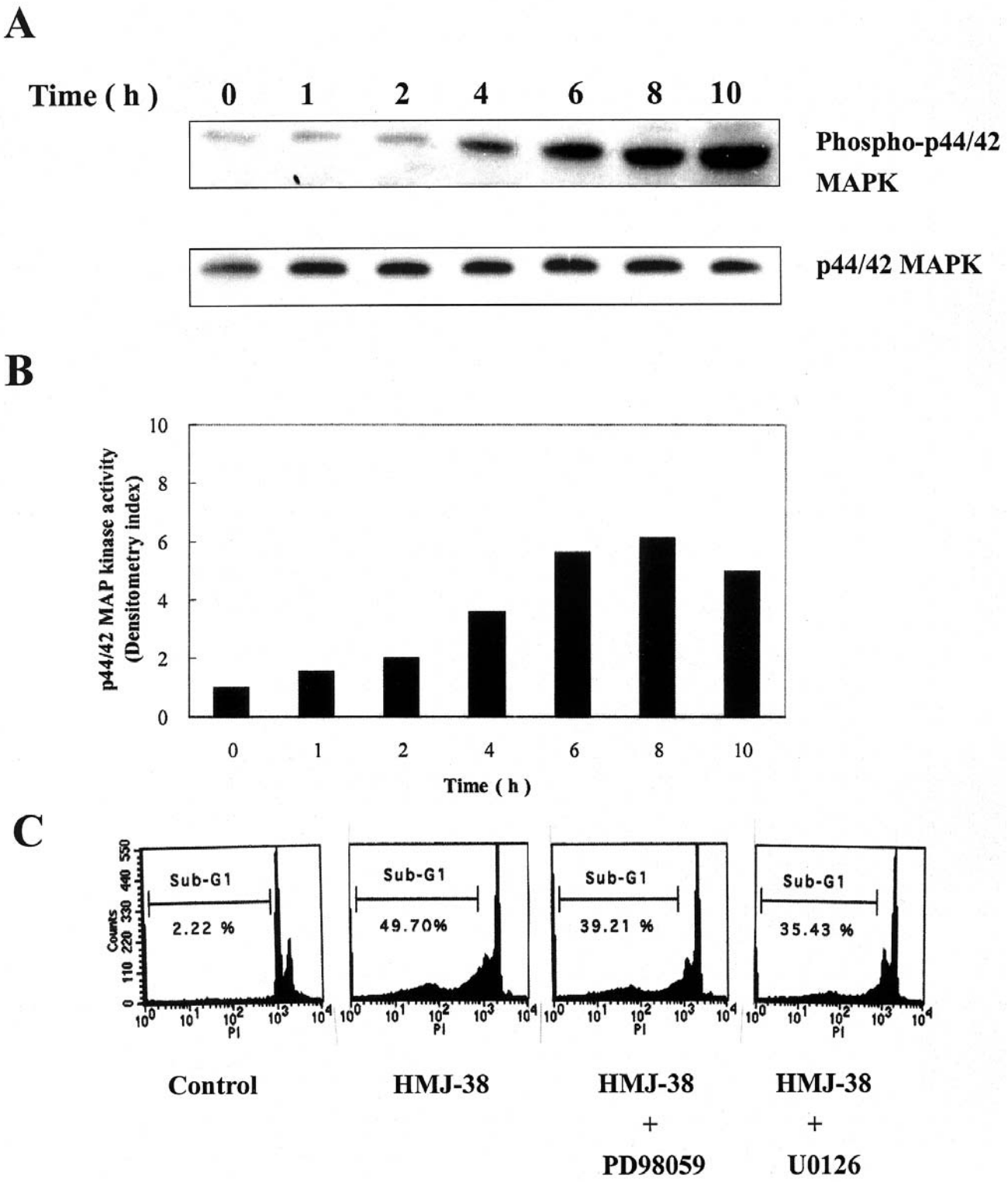

Figure 6. *HMJ-38 activated ERK cascade during apoptosis. (A) HMJ-38 up-regulated protein level of phospho-ERK. HL-60 cells were treated with HMJ-38 and harvested at the time indicated. Total cell extracts were subjected to Western blot analysis for ERK and phospho-ERK protein. (B) ERK kinase assay. The total cell extracts were immunoprecipitated with phospho-ERK antibody. The immunoprecipitates were incubated with ATP and Elk fusion protein. The eluted proteins were separated by SDS-PAGE and immunoblotted with phospho-Elk specific antibody. (C) PD98059 and U0126 reduced HMJ-38-induced apoptosis analyzed by DNA content analysis after 24 hours of treatment.*

previously described (27). Briefly, protein extract was incubated with immobilized dual phospho-ERK kinase antibody at 4 ˚C overnight. The lysate was centrifuged at 13,000 rpm and the immunoprecipitated phosphor-ERK was soaked with kinase buffer. Elk fusion protein (2 μg) and 200 μM ATP were added to each sample and then incubated at 30˚C for 30 min. The reaction was stopped by adding 30 μl of SDS sample buffer and heating to 100˚C for 5 min. The eluted proteins were separated by SDS-PAGE and then transferred onto PVDF membrane. The specific protein band was detected by using antibody specific to phospho-Elk and anti-mouse antibody conjugated with HRP. ECL kit was used to visualize the detected protein bands.

Results

Effects of HMJ-38 on cell viability and cell cycle progression in HL-60 and PBMC. We treated HL-60 cells, PBMC and PHA-stimulated PBMC with HMJ-38 at different concentrations (5 to 100 μM). The number of viable cells was counted by PI exclusion method 24 h later. As shown in Figure 2A, HMJ-38 exerted a dose-dependent anti-proliferative action on HL-60 cells. The concentration required to inhibit growth by 50% (IC_{50}) for HL-60 was approximately 4.48 μM. The cytotoxic effect of 24-h treatment with HMJ-38 on unstimulated and PHA (5μg/ml)-stimulated PBMC were lower than that in HL-60 cells. To investigate the mechanisms by which HMJ-38 induced a distinct difference of cytotoxicity between HL-60 and PBMC, we cultured HL-60 cells or PBMC for various time lengths with 5 μM HMJ-38 and analyzed DNA content by flow cytometry. As shown in Figure 2B, HMJ-38 induced a time-dependent accumulation of the G2/M population in HL-60 cells, and then the cells underwent apoptosis. Nevertheless, either with or without HMJ-38 treatment, PBMC cells still remained at the G0/G1-phase (96.83% G0/G1 at 48 h and 96.46% G0/G1 at 72 h). We further analyzed the effect of HMJ-38 on mitogen-activated PBMC. After 48 h of PHA-stimulation, the population of PBMC at both S and G2/M gradually increased (30.41% S, 13.83% G2/M). HMJ-38 gradually but significantly affected the cell cycle of PHA-activated PBMC (18.13% S and 22.90% G2/M at 48 h, 20.29% S and 33.65% G2/M at 72 h). These data indicate that HMJ-38 was less toxic in PBMC than in HL-60 cells.

Effect of HMJ-38 on the expression and activity of the G2/M-phase involved proteins. To reveal the mechanisms of HMJ-38-induced G2/M arrest, we investigated the expression of CDK1, cyclin B and the CDK1 activity. After exposure to 5 μM HMJ-38, HL-60 showed an increase in the protein level of CDK1 and cyclin B, peaking at 6-8 h (Figure 3A). Similarly, enzymatic assays on HMJ-38-treated HL-60 cells showed a transient increase in the activity of CDK1, peaking around 4-6 h after HMJ-38 treatment (Figure 3B). To determine the mechanism by which CDK1 was regulated by HMJ-38 in HL-60 cells, we examined the upstream regulators of CDK1. As shown in Figure 3A, HMJ-38 induced a time-dependent increase of $p21^{waf/cip}$, Chk1 and Wee l, whereas a decrease of Cdc25C was observed at protein levels. Thus, these results suggest that the CDK1/cyclin B expression and activity were transiently up-regulated in response to HMJ-38 and then down-regulated by Chk1, Wee l, Cdc25C and $p21^{waf/cip}$ in HL-60 cells.

HMJ-38-induced apoptosis in HL-60 cells by down-regulation of Bcl-2, up-regulation of Bax protein and accumulation of cytosolic cytochrome c. Cell cycle analysis revealed that HMJ-38 induced G2/M arrest followed by the appearance of sub G1 nuclei. Furthermore, treatment with HMJ-38 also resulted in DNA fragmentation as evidenced by the formation of DNA ladder on agarose gels (Figure 4A), which was another hallmark of cells undergoing apoptosis. To examine whether the mitochondria-mediated apoptotic pathway involved in HMJ-38-induced apoptosis, we examined the expression levels of these Bcl-2 family proteins and cytosolic cytochrome *c*. As shown in Figure 4B, exposure of HL-60 cells to 5 μM HMJ-38 resulted in a marked time-dependent increase in Bax and a decrease in Bcl-2 protein levels. In addition, the protein level of cytosolic cytochrome c increased in a time-dependent manner after addition of HMJ-38 (Figure 4C). These results suggest that a mitochondrial pathway mediates HMJ-38-induced apoptotic response.

HMJ-38-induced apoptosis is mediated by the activation of caspase-9 and caspase-3. One of the major pathways of apoptosis is mediated by serial activation of caspases. Therefore, we investigated the HMJ-38-treated HL-60 cells for the caspase-9, caspase-8 and caspase-3 activities by Western blot analysis and chromogenic enzymatic assay. Both caspase-9 and caspase-3 activities increased 8 h after HMJ-38 treatment (Figure 5A, 5B), whereas the activity of caspase-8 was not affected (data not shown). Moreover, pre-incubation with z-LEHD-fmk, an inhibitor of caspases-9, strongly reduced the HMJ-38-induced caspase-9 and caspase-3 activities and apoptosis in HL-60 cells (Figure 5C). The time course of cytosolic cytochrome c accumulation correlated well with the proteolytic activation of caspase-9 and caspase-3 and cleavage of PARP (Figure 4C, 5A, 5B). However, pre-incubation with z-VAD-fmk, a broad-spectrum inhibitor of caspase, did not prevent the HMJ-38-triggered accumulation of cytosolic cytochrome c. These results suggest that HMJ-38-induced caspase-9 and caspase-3 activation is downstream of the accumulation of cytosolic cytochrome c.

HMJ-38-induced apoptosis is mediated by the ERK cascade in HL-60 cells. Many intracellular signaling cascades have been reported to take part in anti-mitotic agent-induced cell cycle arrest or apoptosis. We approached this possibility by

respectively studying the effect of the specific inhibitors of PKA, PKB, PKC, MEK and p38MAPK (H89, Wortmannin, RO32-0432, PD98059, U0126 and SB203580) on HMJ-38-induced G2/M arrest and apoptosis in HL-60 cells. HL-60 cells were treated either with 5 μM HMJ-38 alone or with 5 μM HMJ-38 plus one of the aforementioned inhibitors. The DNA content was then analyzed by flow cytometry after 24 h of treatment. Among these inhibitors, only the ERK pathway specific inhibitors, PD98059 and U0126, significantly inhibited HMJ-38-induced apoptosis (Figure 6C). We also examined the ERK activity of HL-60 cells upon exposure to HMJ-38. HMJ-38 increased both the phosphorylation level and enzymatic activity of ERK, without affecting its protein level (Figure 6A, 6B). However, none of these inhibitors blocked HMJ-38-induced G2/M arrest (Figure 6C). These data suggested a role of ERK activation in HMJ-38-induced apoptosis in HL-60 cells.

Discussion

Interference with microtubule assembly/disassembly represents an important concept in anticancer drug mode of action. Over the past decades, several microtubule- binding inhibitors have been developed as clinical anticancer agents (28). HMJ-38 was developed in the hope of reducing the negative side-effects associated with other microtubule inhibitors. Our data showed that 5 μM HMJ-38 significantly inhibited the proliferation of HL-60 human leukemia cells. However, HMJ-38 has much less cytotoxicity on PBMC than on HL-60 cells. In addition, HMJ-38 inhibited the proliferation of other human leukemia cell lines (U937 and K562) and mouse leukemia cell lines (Raw 264.7 and WEHI-3) (data not shown). In contrast, it exerts low cytotoxicity on normal human skin cell lines, WS-1 and Detroit 551 (IC_{50} > 200 μM). These data suggest that HMJ-38 represents a promising candidate as an anti-cancer agent with low toxicity to normal cells.

When cells enter mitosis, many microtubule interacting agents may interfere with the dissociation of chromosomes, thus activating cell cycle checkpoints and inducing G2/M arrest and/or apoptosis. The G2/M checkpoint plays an important role in providing time for DNA repair, whereas apoptosis may function to remove irreparably damaged cells (4). Because HMJ-38 is a microtubule-binding agent, we focused its cellular responses on the G2/M-phase in this study. Our data showed that HMJ-38 started to induce G2/M-phase arrest at 6 h of treatment, but sub-G1 nuclei appeared at 12 h. These results indicate that HMJ-38-induced G2/M arrest occurred before the onset of apoptosis. The CDK1/cyclin B complex is one of the major regulators governing the G2 to M progression or apoptosis. The exact regulation of the CDK1/cyclin B activity is cardinal for normal cell cycle and is subject to multiple regulatory steps (29). Recent reports have shown that G2/M checkpoints can stimulate Wee 1, the so-called checkpoint kinases (Chk1 and Chk2, which inactivate Cdc25C) and CDK inhibitor ($p21^{waf/cip}$) to inactivate CDK1, and prevent cells from entering mitosis (8-11). Our data showed that HMJ-38 induced a transient increase both in the protein levels and in the enzymatic activity of CDK1 and cyclin B at 4-6 h of treatment (Figure 3). When the CDK1/cyclin B activity decreased 6 h after treatment, the protein levels of its upstream regulators, $p21^{waf/cip}$, Chk1 and Weel, increased, whereas Cdc25C decreased. These results are similar to previous reports regarding the regulation of CDK1/cyclin activity (29). Our data suggest that Chk1, Weel, Cdc25C phosphatase and $p21^{waf/cip}$ proteins are involved in the regulation of CDK1/cyclinB in the HMJ-38-treated HL-60 cells. The observed transient increase of CDK1/cyclin B activity, followed by the appearance of apoptotic nuclei (sub G1 nuclei) and DNA fragmentation, suggested that CDK1 activity might be involved in the HMJ-38-induced apoptosis (Figure 1B, 2). The increase of CDK1 activity has been suggested to support both promotion and inhibition of apoptosis (30). This possibility may be elucidated by directly blocking CDK1 activity or blocking its downstream targets (such as Bad, Bcl-2 and survivin) (30-32).

Activation of the caspase cascades is the major mechanism which promotes apoptosis in response to death-inducing signals from cell surface receptors, mitochondria, or endoplasmic reticulum stress (33-35). In this study, we observed the induction of caspase-9 and caspase-3 specific activities (at 12 h of treatment) before the onset of DNA fragmentation (16 h treatment), and the specific cleavage of PARP by caspases-3-like activity (12 to 24 h treatment) by HMJ-38 (Figures 4, 5). We also found that caspase inhibition prevented HMJ-38-induced apoptosis. Furthermore, we detected loss of mitochondria membrane potential in HMJ-38-treated HL-60 cells (data not shown) and release of mitochondrial cytochrome *c* to cytosol (Figure 4) after 12 h of treatment. These results suggest that HMJ-38-induced apoptosis is mediated through the activation of caspase cascades and is mitochondria-dependent. Recent reports have shown that, during apoptosis, some pro-apoptotic factors, including procaspase and caspase-independent factors such as apoptosis-inducing factors (AIF), can be released from mitochondria into cytosol (36). Our results do not exclude the possibility of involvement of other caspases or caspase-independent factors in the HMJ-38-induced apoptosis. Bcl-2 family members have been reported to interfere in the regulation of mitochondria-mediated apoptotic pathways. Some reports also demonstrated that Bcl-2 and Bax locate in the mitochondrial outer-membrane and the Bcl-2/Bax ratio regulates the release of mitochondrial cytochrome *c* to cytosol (37). Our results are consistent with the published observations as are evidenced by the up-regulation of pro-

apoptotic Bax protein and the down-regulation of anti-apoptotic Bcl-2 protein during HMJ-38-induced apoptosis.

MAPK cascades have been reported to take part in anticancer agent-induced apoptosis (38). Our results showed that HMJ-38-induced apoptosis, but not G2/M arrest, is mediated by the ERK pathway on HL-60 cells (Figure 6). Similarly, Bacus *et al.* have demonstrated that Taxol-induced apoptosis in MCF-7 cells is mediated by ERK activity (39, 40). Moreover, recent reports showed that almost all microtubule-active drugs (*Vinca* alkaloids, taxol, podophyllotoxin, colchicines, epothilones and nocodozole) induce phosphorylation of Bcl-2 protein, which results in inactivation of Bcl-2 and loss of anti-apoptotic function, whereas anti-metabolites and alkylation agents do not (37). Our preliminary results indicated that Bcl-2 phosphorylation by ERK is involved in HMJ-38-induced apoptosis (data not shown).

The biochemical mechanism by which altered microtubule dynamics leads to cytotoxicity are not well defined. It has been proposed that prolonged mitotic arrest stimulates apoptosis (37), but the biochemical pathway linking mitotic arrest and apoptosis remains unclear. The ability to inhibit tubulin polymerization *in vitro* suggested that HMJ-38 might exert the same inhibitory effect *in vivo* as vinca alkaloids (such as colchicine, vincristine and vinblastine) do. They can inhibit microtubule assembly and block the cell cycle progression, which leads to cell death. Further experiments are required to prove that the cytotoxicity of HMJ-38 correlates with its effect on microtubules. In addition, the possibility that targets other than microtubules or other secondary pathways leading to cell death may exist, cannot be excluded.

In conclusion, HMJ-38 showed significant cytotoxicity against cancer cells. However, HMJ-38 is less toxic for PBMC and normal cell lines. HMJ-38 induced G2/M arrest followed by caspase-mediated apoptosis on HL-60 cells. The HMJ-38-induced apoptosis was ERK-dependent. Taken together, these findings provide important new insights into the possible molecular mechanisms of the anti-cancer activity of HMJ-38.

Acknowledgements

This work was supported by grant CMC-91-M-13 from the China Medical University, Taiwan, Republic of China.

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Received December 4, 2003
Revised February 18, 2004
Accepted April 1, 2004