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Cinnamaldehyde-induced apoptosis in human PLC/PRF/5 cells through activation of the proapoptotic Bcl-2 family proteins and MAPK pathway

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Abstract

Cinnamaldehyde (Cin) has been shown to be effective in inducing apoptotic cell death in a number of human cancer cells. However, the intracellular death signaling mechanisms by which Cin inhibits tumor cell growth are poorly understood. In this study, we investigated the effect of mitogen-activated protein kinases (MAPKs) inhibitors [namely SP600125 (a specific JNK inhibitor), SB203580 (a specific p38 inhibitor) and PD98059 (a specific ERK inhibitor)] on the stress-responsive MAPK pathway induced by Cin in PLC/PRF/5 cells. Trypan blue staining assay indicated that Cin was cytotoxic to PLC/PRF/5 cells. Cin caused cell cycle perturbation (S-phase arrest) and triggered apoptosis as revealed by the externalization of annexin V-targeted phosphatidylserine and accumulation of sub-G1 peak. It down-regulated the Bcl-2 and Mcl-1 expression, and up-regulated Bax protein in a time-response manner. Treatment with 1 μM Cin resulted in an activation of caspase-8 and cleavage of Bid to its truncated form in a time-dependent pattern. JNK, ERK and p38 kinases in cells were activated and phosphorylated after Cin treatment. Pre-incubation with SP600125 and SB203580 markedly suppressed the effect of Cin-induced apoptosis, but not PD98059. Both SP600125 and SB203580 significantly prevented the phosphorylation of JNK and p38 proteins, but not ERK. These results conclude that Cin triggers apoptosis in

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PLC/PRF/5 cells could be through the activation of pro-apoptotic Bcl-2 family (Bax and Bid) proteins and MAPK signaling pathway.

Keywords: Cinnamaldehyde; Bcl-2 family; MAPK; Apoptosis; PLC/PRF/5 cells

Introduction

Programmed cell death or apoptosis, is critical not only during development and regulation of cellular homeostasis but also in the pathogenesis of a variety of diseases including cancer, autoimmune disease, stroke and neurodegenerative disorders (Thompson, 1995; Meier et al., 2000).

Cinnamaldehyde (Cin), a bioactive compound isolated from the stem bark of *Cinnamomum cassia* Presl (Lauraceae), has been widely used in folk medicine as anticancer (Ka et al., 2003), antibacterial (Chang et al., 2001), anti-mutagenic (Shaughnessy et al., 2001), immunomodulatory (Koh et al., 1998), and as remedies for treating other diseases (Perry, 1980). Studies have demonstrated that Cin induced the generation of reactive oxygen species (ROS), reduction of mitochondrial membrane potential, release of cytochrome *c* and activation of caspase activity in human leukemia HL-60 cells (Usta et al., 2002; Ka et al., 2003). However, the signaling pathways responsible for cell death following Cin administration remain unclear.

Bcl-2 family proteins play a pivotal role in controlling cell life and death. They are major regulators of the apoptotic process, and comprise pro-apoptotic (Bax, Bak, Bid, Noxa etc) and anti-apoptotic (Bcl-2, Bcl-$_{XL}$, Mcl-1, Bcl-w etc) molecules (Adams and Cory, 1998; Borner, 2003). Bid acts as one of the links between the Bcl-2 family members and the caspases. In cancer treatment, most chemotherapeutic agents induced apoptosis involves the cleavage of Bid by caspase-8, which then causes the release of cytochrome *c* from mitochondria, the cytochrome *c* together with Apaf-1 activates caspase-9, and the latter activates caspase-3 and consequently resulting in cell apoptosis (Luo et al., 1998; Anto et al., 2002).

The mitogen-activated protein kinases (MAPKs) consist of three subtypes including the extracellular signal-regulated kinases (ERK), stress-activated protein kinases (SAPKs)/c-Jun NH_2-terminal kinases (JNK) and p38 kinases (Yang et al., 2003). In mammalian cells, MAPKs can transduce a diverse extracellular stimulus (including mitogenic growth factors, hormones, cytokines, environmental stresses and chemotherapy agents) to the nucleus via kinase cascades to regulate cell survival, differentiation and apoptosis (English et al., 1999). In general, JNK and p38 are activated by proinflammatory cytokines and environmental stresses such as UV irradiation, heat, hydrogen peroxide and DNA damage. They also promotes cell apoptosis. ERK is mainly activated by growth factors, and was reported to associate with cell proliferation and differentiation (Xia et al., 1995; Ichijo, 1999). However, a growing number of evidence has suggested that activation of ERK can also result in the cell death (Julio et al., 2001; Yu et al., 2001).

In this study, our aims were: (i) to evaluate the antihepatoma activity of Cin, and its effects on cell growth and apoptosis in human hepatoma PLC/PRF/5 (CD95-negative) cells; (ii) to examine the role of Bcl-2 family proteins (Bax, Bid, Bcl-2 and Mcl-1), caspase-8, and the MAPK signal pathway in the regulation of Cin-mediated apoptosis; and (iii) to study the effects of JNK (SP600125), p38 (SB203580) and ERK (PD98059) inhibitors on the MAPK pathway.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), penicillin, streptomycin, tryspin-EDTA, trypan blue, caspase-8 substrate (Ac-IETD-pNA) and anti-β-actin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from GIBCO BRL (Gaithersburg, MD). JNK (SP600125), p38 (SB203580) and ERK (PD98059) inhibitors were purchased from Calibiochem (San Diego, CA, USA). The anti-Bax, anti-Bcl-2, anti-Bid, anti-Mcl-1, anti-JNK, anti-phospho-JNK (pT183/pY185), anti-p38, anti-phospho-p38 (pT180/pY182), anti-ERK, anti-phospho-ERK (pT202/pY204) and anti-rabbit IgG bodies were purchased from PharMingen (San Diego, CA, USA). Anti-mouse IgG antibody was from Promega (Madison, WI, USA).

Cell cultures and drug preparation

The PLC/PRF/5 cells (ATCC CRL 8024; hepatitis B surface antigen, HBsAg [+]) were obtained from the American Type Culture Collection (Rockville, MD, USA), and were grown in 90% DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. They were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

The Cin stock solution was prepared in DMSO and was stored at −20 °C until use. The concentrations used for the study were 0.1, 0.5 and 1 μM, which were freshly prepared for each experiment with a final DMSO concentration of 0.1%.

Cell viability assay

For Cin-induced viability assay, cells were seeded at a density of $1x10^5$ cells/well onto 12-well plates with 0.1% DMSO (control) or 0.1, 0.5, 1 and 5 μM Cin. In the experiment, cells were pretreated with each inhibitor (20 μM JNK or 25 μM p38 or 50 μM ERK) for 1 h before adding Cin. After 24 h incubation, the number of viable cells was determined by staining cell populations with trypan blue. The number of unstained (viable) cells was counted using a hemocytometer.

Detection of Cin-induced apoptosis

The annexin V-FITC Apoptosis Detection kit (Roche Diagnostics GmbH, Germany) was used to label the externalized phosphatidylserine according to the manufacturer's protocol. The analysis was performed with a flow cytometer (Coulter Epics Elite ESP; Miami, FL, USA) equipped with a 488-nm argon laser. Approximately 10,000 cells were evaluated for each sample. Gating of control nonapoptotic populations (cells treated with 0.1% DMSO) was used as a reference to compare with Cin-treated groups.

Flow cytometric detection of cell cycle

After 6, 12 and 24 h of incubation, cells were treated with 0.1% DMSO (control), 0.5 and 1 μM Cin. Floating and adherent cells were then collected. The cells in suspension were fixed with 70% ice-cold

methanol and then transferred to the freezer until use. After washing with phosphate buffered saline (PBS), cells were stained with 50 μg/ml propidium iodide in the presence of 25 μg/ml RNase A at 37 °C for 30 min. A minimum of 10,000 cells per sample was collected. To estimate the percentage of each phase in cell cycle, the DNA histograms were analyzed by Multicycle software (Phoenix Flow Systems, San Diego, CA., USA).

Assay of caspase -8

The enzymatic activity of caspase-8 induced by Cin was assayed according to the manufacturer's protocol. To evaluate the activity of caspase-8, cell lysates were prepared after their respective treatment with control (0.1% DMSO) and 1 μM Cin for 0, 6, 12 and 24 h at 37°C. Assays were performed in 96 well microtitre plates by incubating 100 μg protein of cell lysate per sample in 100 μl of reaction buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl and 10% glycerol] containing 2 μl of caspase-8 substrate (Ac-IETD-pNA). Lysates were further incubated at 37 °C for 4 h. The sample was measured with an ELISA reader at the absorbance of 405 nm (Anthos 2010, Austria).

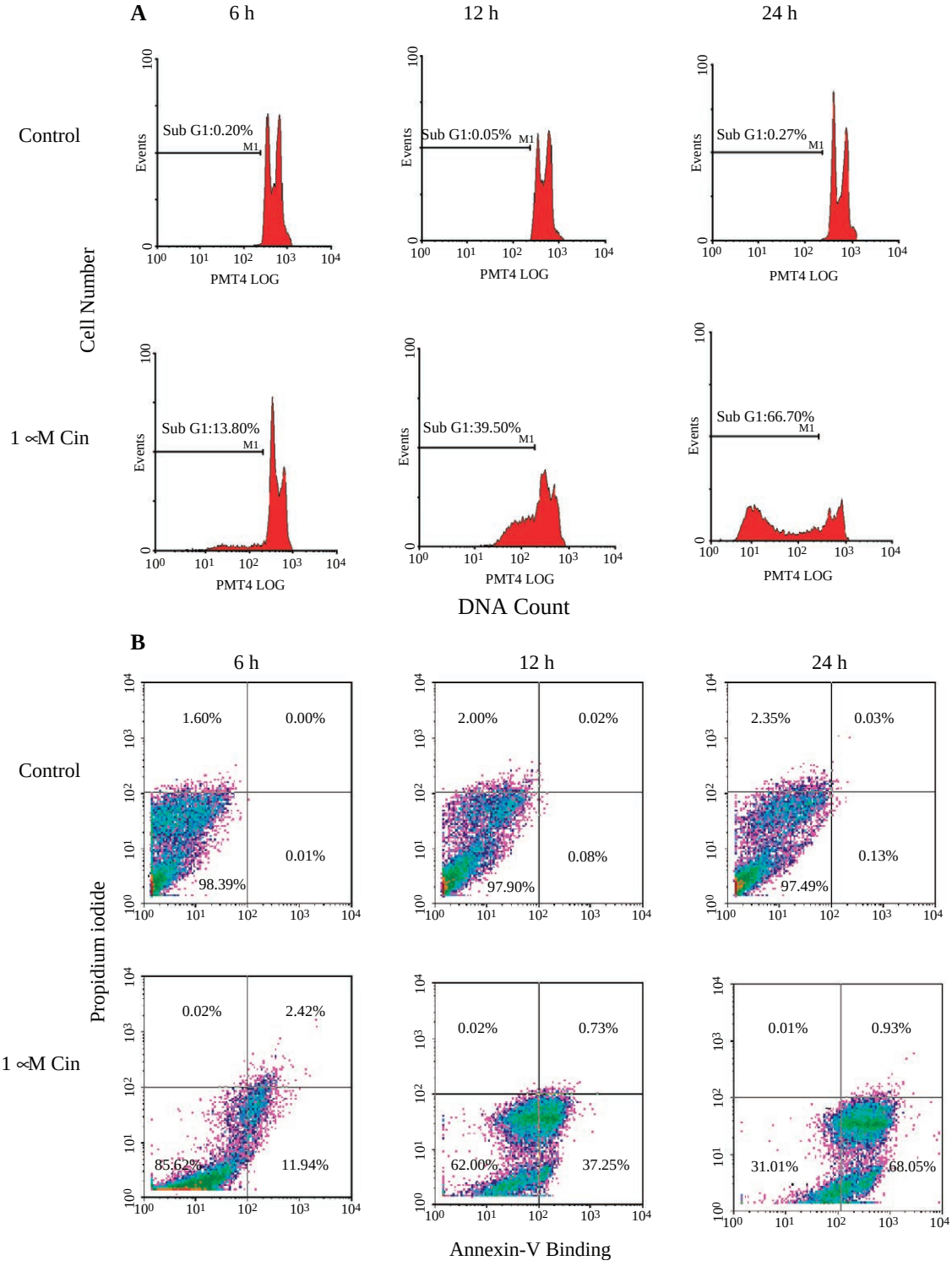
Western immunoblot analysis

Cells were harvested and lysed in ice-cold buffer (10 mM Tris-HCl, pH 7.5, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM sodium orthovanate and 120 mM sodium chloride) containing 1mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 1 μg/ml aprotonin (Sigma Chemical Co., St. Louis, MO, USA). Lysates were centrifuged at 10,000 × g for 10 min. Equal amounts of lysate protein (50 μg/lane) were loaded onto SDS-polyacrylamide gels and electrophoretically transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). After inhibiting the nonspecific binding sites with 5% (w/v) skim milk in 0.1% (v/v) Tween 20 containing PBS (PBST) for 1 h at room temperature, the membrane was incubated with the specific primary antibodies [namely anti-Bax (1:250), anti-Bcl-2 (1:500), anti-Bid (1:2000) and anti-Mcl-1 (1:500)], MAPK primary antibodies [namely anti-JNK (1:250), anti-phospho-JNK (pT183/pY185) (1:250), anti-p38 (1:5000), anti-phospho-p38 (pT180/pY182) (1:2500), anti-ERK (1:5000) and anti-phospho-ERK (pT202/pY204) (1:1000)] and anti-β-actin (1:5000) antibodies in 5% (w/v) skim milk in PBST for 1 h at room temperature. Antibody recognition was detected with the respective secondary antibody, either anti-

Table 1
Effect of cinnamaldehyde (Cin) on PLC/PRF/5 cell viability

Treatment (μM)	Cell viability (%)			
	0 h	6 h	12 h	24 h
0	100.0 ± 0.00[a]	100.0 ± 0.00[a]	100.0 ± 0.00[a]	100.0 ± 0.00[a]
0.1	100.0 ± 0.00[a]	100.0 ± 0.00[a]	100.0 ± 0.00[a]	87.0 ± 1.00[b]
0.5	100.0 ± 0.00[a]	100.0 ± 0.00[a]	55.7 ± 2.62[b]	40.0 ± 1.63[c]
1.0	100.0 ± 0.00[a]	89.0 ± 0.10[b]	47.7 ± 2.05[c]	35.3 ± 0.47[d]
5.0	99.0 ± 0.33[a]	80.0 ± 0.70[c]	46.0 ± 1.63[c]	4.3 ± 0.50[e]

After Cin treatment, cell numbers were estimated by trypan blue staining method. Values are means ± S.D. of three independent experiments. Values in the same column with different letters were significantly difference at $P < 0.05$ as analyzed by Duncan's multiple range tests.


A
6 h
12 h
24 h
Control
1 ∝M Cin
Cell Number
Sub G1:0.20%
Sub G1:0.05%
Sub G1:0.27%
Sub G1:13.80%
Sub G1:39.50%
Sub G1:66.70%
M1
Events
PMT4 LOG
DNA Count
B
6 h
12 h
24 h
Control
1 ∝M Cin
Propidium iodide
1.60%
0.00%
98.39%
0.01%
2.00%
0.02%
97.90%
0.08%
2.35%
0.03%
97.49%
0.13%
0.02%
2.42%
85.62%
11.94%
0.02%
0.73%
62.00%
37.25%
0.01%
0.93%
31.01%
68.05%
Annexin-V Binding

mouse IgG or anti-rabbit IgG antibody linked to the horseradish peroxidase. Antibody-bound proteins were detected by the ECL western blotting analysis system (Amersham, Aylesbury, UK). The expression of β-actin was used as a control.

Statistical analysis

Data were presented as mean ± standard deviations (S.D.) from three independent experiments. Values were evaluated by one way ANOVA, followed by Duncan's multiple range tests using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Control and treatment groups were compared among themselves using Student's *t*-test. Differences were considered significant when *P*-value was < 0.05.

Results

Effects of Cin on the survival of PLC/PRF/5 cells

To determine the effects of Cin on cultured human hepatoma PLC/PRF/5 cells, cells were treated with 0, 0.1, 0.5, 1 and 5 μM Cin for 0, 6, 12 and 24 h. After Cin treatment, cell viability was evaluated by trypan blue staining assay. As shown in Table 1, Cin inhibited the proliferation of PLC/PRF/5 cells in a dose- and time-dependent pattern. After 24 h of treatment, 1 μM Cin caused nearly a 65% inhibition of cell growth as compared to the control.

Induction of apoptosis by Cin

To further confirm the finding that Cin-induced apoptosis, PLC/PRF/5 cells were stained with annexin V-FITC and propidium iodide, and subsequently analyzed by flow cytometry. As indicated by FACS analysis, the proportion of annexin V-staining cells was increased in 1 μM Cin-treated cells (Fig. 1A and B). The percentage of sub-G1 vs. annexin V-staining cells were 13.80±0.32% vs 11.94±1.60% for 6 h; 39.50±4.70% vs 37.25±2.50% for 12 h and 66.70±3.90% vs 68.05±3.20% for 24 h, respectively (Table 2). PLC/PRF/5 cells treated with Cin exhibited a significant increase in the number of apoptotic cells in a time-dependent manner (6, 12 and 24 h).

Flow cytometric detection of cell cycle

The effect of Cin on PLC/PRF/5 cell cycle was investigated by flow cytometry. Results showed that cells treated with 1 μM Cin displayed a time-dependent accumulation of the sub-G1 region (i.e. loss of fragmented DNA), an increase from 13.80% to 66.70% for 6 h to 24 h, respectively (Fig. 1A). After 6, 12 and 24 h of treatment, 1 μM Cin induced cell cycle arrest in

Fig. 1. Induction of apoptosis by Cin in PLC/PRF/5 cells. Effect of control (0.1% DMSO) and 1 μM Cin treatment on PLC/PRF/5 cell apoptosis at 6, 12 and 24 h. (A) Effect of Cin on cell cycle progression. (B) Flow cytometric analysis of annexin V-FITC and propidium iodide double stained cells.

Table 2
Effect of cinnamaldehyde (Cin) on incidence of apoptosis in PLC/PRF/5 cells

Treatment	%	
	Sub-G1 DNA	Annexin V
6 h		
Control	0.20 ± 0.00	0.01 ± 0.00
1 μM Cin	13.80 ± 0.32*	11.94 ± 1.60*
12 h		
Control	0.05 ± 0.02	0.08 ± 0.01
1 μM Cin	39.50 ± 4.70*	37.25 ± 2.50*
24 h		
Control	0.27 ± 0.03	0.13 ± 0.00
1 μM Cin	66.70 ± 3.90*	68.05 ± 3.20*

Values are mean ± S.D. of three independent experiments. The DNA content (sub-G1 peak) and the binding of annexin V-FITC to the plasma membrane were analyzed by flow cytometry. The asterisk indicates a significant difference between control and Cin-treated cells at $*P < 0.01$ as analyzed by Student's *t*-test.

the S phase and resulted in a decline in the percentage of cells in the G2/M phase (Table 3). These findings provided preliminary evidence that Cin may activate the programmed cell death machine.

Cin induces activation of caspase-8

Cell lysates were treated as described in method and materials. After 6 h of Cin treatment, a marked increase in the caspase-8 activity (190.10 ± 4.02%) was noted, it reached the maximum (460.00 ± 13.55%) after 24 h of Cin treatment (Fig. 2).

Table 3
Effects of cinnamaldehyde (Cin) on PLC/PRF/5 cell cycle distribution

Treatment	% Cell distribution		
	G0/G1	S	G2/M
6 h			
Control	50.60 ± 1.90	15.90 ± 0.80	33.50 ± 1.30
1 μM Cin	55.00 ± 1.02*	32.70 ± 1.85**	12.30 ± 0.50**
12 h			
Control	48.00 ± 1.30	22.60 ± 1.50	29.40 ± 1.00
1 μM Cin	43.90 ± 1.90*	35.60 ± 1.02*	20.50 ± 0.10**
24 h			
Control	53.90 ± 1.20	31.90 ± 0.75	14.20 ± 0.85
1 μM Cin	45.40 ± 1.74*	42.10 ± 1.00*	12.50 ± 0.62

Values are mean ± S.D. of three independent experiments. The control is 0.1% DMSO. The asterisk indicates a significant difference between control and Cin-treated cells at $*P < 0.01$ and $**P < 0.005$ as analyzed by Student's *t*-test.

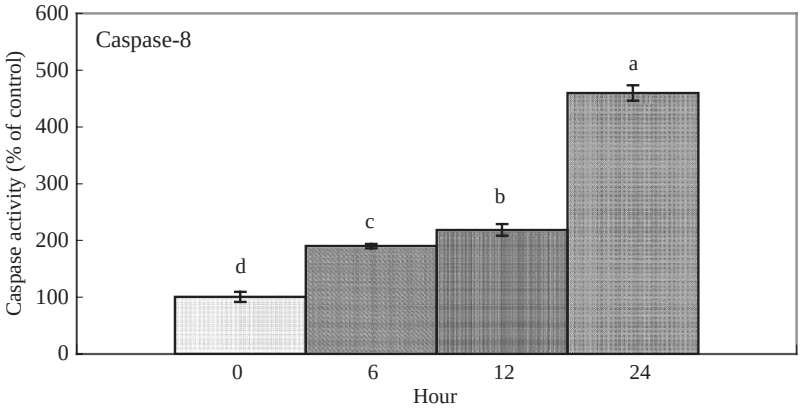


Fig. 2. The activation of caspase-8 by Cin in PLC/PRF/5 cells. Extracts from untreated or Cin-treated cells were assay for proteolytic cleavage of caspase-8 substrate. Data are mean ± S.D. of three independent experiments. Values with different letters were significantly difference at $P < 0.05$ as analyzed by Duncan's multiple range tests.

Effect of Cin on Bcl-2 family proteins

The expression levels of the Bcl-2 family proteins in PLC/PRF/5 cells were evaluated after treating with 1 μM Cin for 0, 6, 12 and 24 h. As indicated in Fig. 3, Cin appeared to induce the cleavage of the pro-apoptotic protein (Bid) from 22 kDa to its truncated form t-Bid (15 kDa) after 24 h of treatment. Thus, it is quite likely that Cin activated caspase-8, which led to cleavage of Bid in a time-dependent response (Figs. 2 and 3). Caspase-8-mediated cleavage of Bid leads to the release of cytochrome *c* from the mitochondria, which is an essential step in the apoptotic pathway activated by chemotherapeutic

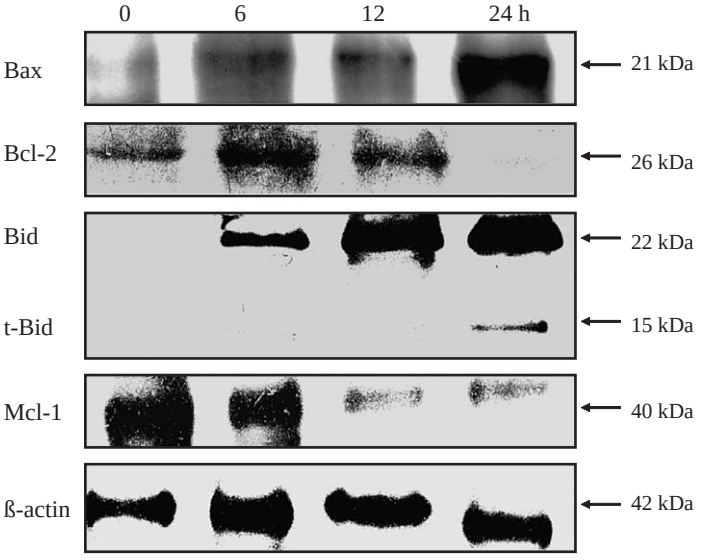


Fig. 3. Effect of Cin on Bcl-2 family proteins. Total cell lysates of PLC/PRF/5 cells treated with 1 μM Cin for the indicated time preriods were analyzed by 12% SDS-PAGE and then immunoblotted with antisera against Bax, Bcl-2, t-Bid, Mcl-1 and β-actin. β-Actin was probed to control for equal loading of protein.

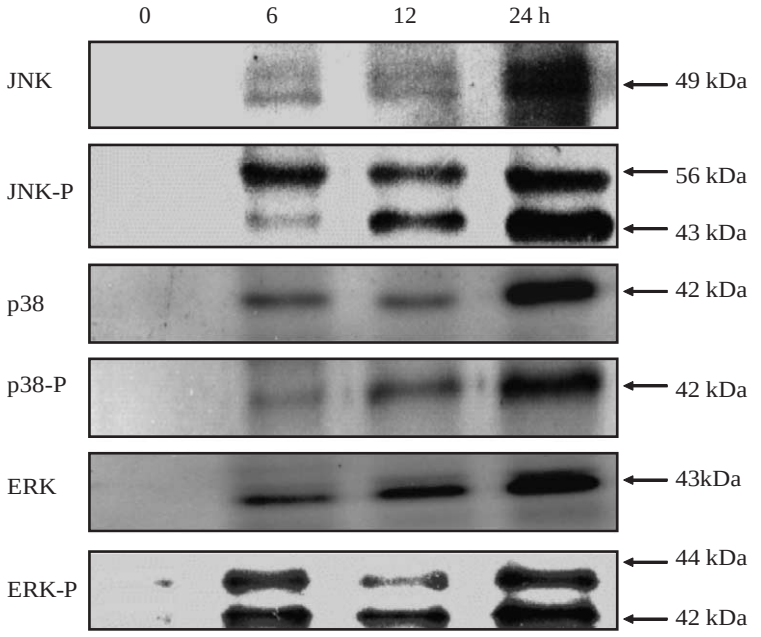


Fig. 4. MAPKs activation and phosphorylation by Cin. PLC/PRF/5 cells were treated with 1 μM Cin. Total cell lysates of cells untreated (control, 0.1% DMSO) and treated with 1 μM Cin for 0, 6, 12 and 24 h were extracted. Western blotting was performed with the activation of JNK, p38 kinase and ERK, and their phosphorylated antibodies.

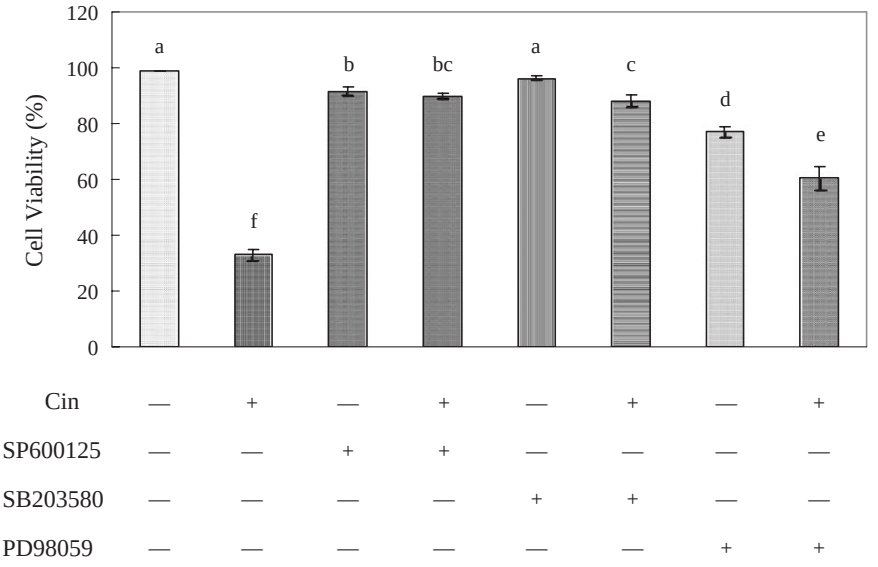


Fig. 5. Effects of JNK, p38 and ERK inhibitors on Cin-induced cell viability. PLC/PRF/5 cells were treated without or with 20 μM JNK inhibitor (SP600125), 25 μM p38 inhibitor (SB203580) and 50 μM ERK inhibitor (PD98059) for 1 h, and then in the presence or absence of 1 μM Cin for 24 h. After incubation, the apoptotic cell number was determined by trypan blue staining assay. Data are presented as means ± S.D. of four independent experiments. Values with different letters were significantly difference at $P < 0.05$ as analyzed by Duncan's multiple range tests.

agents (Anto et al., 2002). Treatment with Cin results in the down-regulation of the anti-apoptotic (Bcl-2 and Mcl-1) proteins and up-regulation of the pro-apoptotic (Bax) protein in a time-dependent fashion (Fig. 3). The expression of Bcl-2 protein had gradually disappeared after 24 h of 1 μM Cin treatment (Fig. 3). These results indicate that the expression levels of Bcl-2 family members modulate Cin-induced cell apoptosis.

Involvement of MAPKs in Cin-induced apoptosis

MAPK signaling pathways have been shown to play an important role in the regulation of apoptosis (Chen et al., 2003a,b; Yang et al., 2003). To characterize the activated and phosphorylated forms of MAPKs (JNK, p38 and ERK) by the immunoblot analysis, we examined the effect of Cin on the MAPK family proteins. As indicated in Fig. 4, all of the three major MAPKs, i.e. JNK, p38 and ERK, were activated and phosphorylated time-dependently in PLC/PRF/5 cells after Cin treatment. The activation and phosphorylation of JNK, p38 and ERK was markedly increased after 24 h of Cin treatment. These results suggest that the death of PLC/PRF/5 cells induced by Cin could be mediated through the MAPK pathways during apoptosis. The relative phosphorylation levels of JNK-P/JNK and ERK-P/ERK were increased in Cin-treated cells, but not the level of p38-P/p38 protein (Fig. 4).

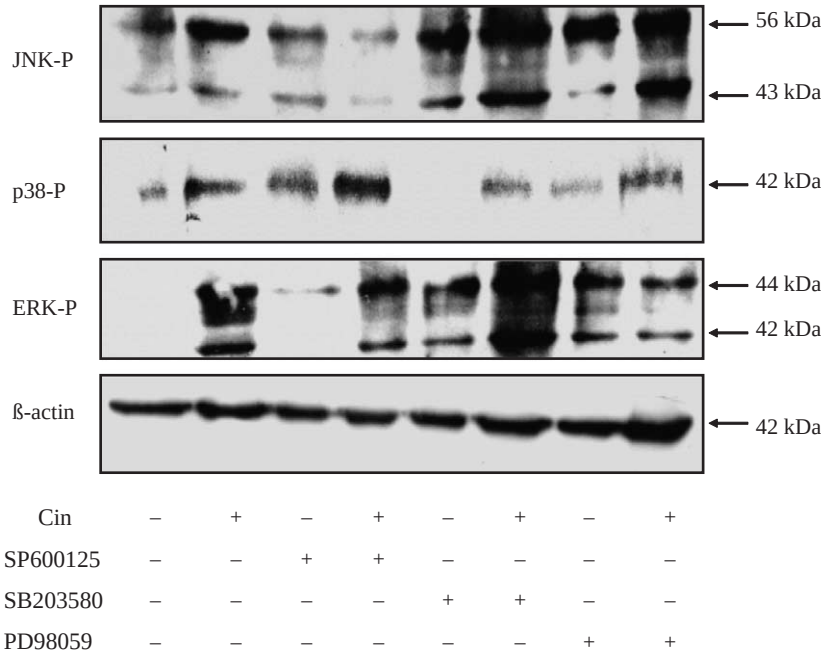


Fig. 6. Effect of MAPK phosphorylation by JNK, p38 and ERK inhibitors (SP600125, SB203580 and PD98059) on Cin-induced apoptosis. PLC/PRF/5 cells were treated without or with 20 μM JNK inhibitor (SP600125), 25 μM p38 inhibitor (SB203580) and 50 μM ERK inhibitor (PD98059) for 1 h, and then in the presence or absence of 1 μM Cin for 24 h. Total cell lysates were then analyzed by Western blotting analysis with respective phospho-MAPK antibodies. β-Actin was probed to control for equal loading of protein.

Effects of MAPK specific inhibitors on Cin-induced cell death

Studies using MAPK inhibitors were carried out to determine whether the activation of MAPKs contributes to Cin-induced cell death in PLC/PRF/5 cells. Fig. 5 shows the effect of JNK inhibitor (SP600125), p38 inhibitor (SB203580) and ERK inhibitor (PD98059) on Cin-induced cell death. Pre-treatment of 20 μM SP600125 and 25 μM SB203580, but not 50 μM PD98059, significantly increased the number of viable cells by Cin.

The specific inhibitors alter MAPK phosphorylation

The role of MAPK inhibitors (SP600125, SB203580 and PD98059) was conducted to determine the influence of MAPK phosphorylation on Cin-induced apoptosis. Pre-treatment with 20 μM SP600125 for 1 h, followed by adding 1 μM Cin resulted in an inhibition of Cin-induced JNK phosphorylation. Co-treatment of SB203580 or PD98059 with Cin led to an increase in JNK phosphorylation. PLC/PRF/5 cells treated with 25 μM SB203580 only resulted in a disappearance of p38 phosphorylation. However, co-treatment of cells with 25 μM SB203580 and 1 μM Cin led to a decrease in the phosphorylation of p38. The phosphorylation of p38 and ERK were significantly increased when cells were co-treated with 20 μM SP600125 and 1 μM Cin. Pre-treatment of cells with 50 μM PD98059 did not block the Cin-induced ERK phosphorylation. However, SB203580 and Cin led to an obvious increased in the ERK phosphorylation (Fig. 6).

Discussion

Cin has been shown to have antitumor activity (Moon and Pack, 1983; Imai et al., 2002; Jeong et al., 2003; Ka et al., 2003). This study further confirmed that Cin inhibited cell proliferation and induced cell apoptosis. Previous report has highlighted the inhibitory effects of the Cin-derivative (CB403) on the cell cycle progression through the arrest of G2/M phase in human leukemia HL-60 cells (Jeong et al., 2003). In this study, however, our focus was on the molecular mechanistic effects of Cin on human PLC/PRF/5 cells. Results showed that treatment with Cin led to the S phase arrest and subsequently caused a decline in the G2/M phase. The Cin-induced apoptosis was confirmed by two independent assays, the annexin V binding and the cell cycle distribution assays. A significant time-dependent increase in the percentage of apoptotic cells was noted in the Cin-treated groups as demonstrated by the expression of annexin V and the phosphatidylserine on the surface of cells, and an accumulation of sub-G1 peak. The appearance of the phosphatidylserine at the outer leaflet of the plasma membrane is regarded as an early marker of apoptosis (Martin et al., 1995). Cin exhibited a significant increase in the appearance of apoptotic cells in a time-dependent fashion.

Treatment of PLC/PRF/5 (CD95-negative) cells with Cin exhibited an up-regulation of Bax protein, and a down-regulation of Bcl-2 and Mcl-1, as well as causing the Bid to cleave upon the activation of caspase-8. Several studies have shown that the Bcl-2 family of proteins is the central of apoptotic regulation (Yu et al., 2003; Choi et al., 2004). Overexpression of Bcl-2, Mcl-1 and Bcl-$_{XL}$ aborts the apoptotic response while increase in the activity of Bax, Bid and Bak promotes cell death (Cory and Adams, 2002). It was reported that Bid is truncated and activated by a cleavage with

caspase-8, which is activated in the TNF-induced apoptotic pathway (Luo et al., 1998). Results of present study displayed that Cin caused an up-expression of Bax and triggering a down-expression of Bcl-2 and Mcl-1 to promote apoptotic activity in PLC/PRF/5 cells. Overexpression of Bcl-2 and Mcl-1 has been found to abolish apoptosis induced by diverse stimuli (Miyake et al., 2001; Wittmann et al., 2003).

Recent evidence indicates that the MAPK family protein kinases, JNK and p38 are important mediators of apoptosis induced by a variety of stress-related stimuli (Chang and Karin, 2001; Hu et al., 2003). The present study demonstrated that JNK, p38 and ERK were activated and phosphorylated in Cin-treated PLC/PRF/5 cells. Early studies correlated JNK and p38 with apoptosis, whereas ERK was implicated in proliferation and protection from apoptosis (Xia et al., 1995). Furthermore, ERK was found to involve in the RRR-α-tocopheryl succinate-induced human breast cancer cell apoptosis (Yu et al., 2001). Norcantharidin was reported to induce an ERK activation in the downstream signal that leads to cell death (Chen et al., 2003b). In the other reports, however, the role of ERK and phosphorylated ERK from pro-apoptotic to pro-survival appeared to depend on the cell type, drug dose, and the status of other signal transduction pathways (Fan and Chambers, 2001). The stress kinases such as JNK, p38 and ERK were reported to activate via phosphorylation by chemotherapy drugs, including caffeic acid phenethyl ester (Lee et al., 2003), epigallocatechin-3-gallate (Chen et al., 2003a) and phenyethyl isothiocyanate (Hu et al., 2003) in many cancer cell lines.

Interesting, treatment with SP600125 and SB203580 significantly attenuated Cin-induced cell death. The SP600125 or SB203580 significantly reduced JNK or p38 phosphorylation. When cells were co-treated with SP600125 and Cin, an increase in ERK phosphorylation was noted. Furthermore, co-treatment of ERK inhibitor and Cin led to an increase in the JNK phosphorylation. This indicated that a cross-talk exist between these two signaling pathways (Chen et al., 2003a). Pre-treatment of SB203580 eliminated Cin-induced p38 phosphorylation. At the same time, the phosphorylation of p38 was slightly increased in the case when cells were co-treated with 20 μM PD98059 and 1 μM Cin. However, treatment of PLC/PRF/5 cells with ERK inhibitor could not inhibit phosphorylation of ERK. In addition, the application of ERK inhibitor also dramatically intensified the damage on PLC/PRF/5 cells causing by Cin. Co-treatment of p38 inhibitor with Cin led to an increase in the ERK phosphorylation.

In conclusion, Cin treatment not only inhibited PLC/PRF/5 cell proliferation, but also causing the cell cycle to arrest in the S phase. Cells treated with Cin exhibited an induction of Bax, a decrease in Bcl-2 and Mcl-1 proteins, an activation of caspase-8 and cleavage of Bid. These events consequently led to cell death. JNK, p38 and ERK were activated and phosphorylated after Cin treatment in a time-dependent pattern. The results suggest that apoptosis induced by Cin could be through the activation of pro-apoptotic Bcl-2 family (Bax and Bid) proteins and MAPK pathway. The influence of Cin on the activation and phosphorylation of MAPKs, as well as on how Cin-initiated chemical signals are converted to apoptotic response remains to be investigated.

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