Vitamin K analog (compound 5) induces apoptosis in human hepatocellular carcinoma independent of the caspase pathway

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A systemic vitamin K analog, compound 5 (Cpd 5), possesses the ability to inhibit cell growth of tumor cells. Therefore, we investigated the effect of Cpd 5 in human hepatocellular carcinoma (HCC) cell lines and evaluated its role in apoptosis. Human HCC cell lines were cultured and treated with Cpd 5. Apoptosis was assessed using DAPI staining and Annexin-V membrane staining. The expression of caspases, XIAP and Bcl-x_L was also investigated. Cpd 5 decreased cell viability in a dose-dependent manner in two HCC cells (HLE and SK-Hep1) containing mutant p53, but not in the HepG2 cell line, which contained wild-type p53. Cpd 5-treated HLE and SK-Hep1 cells showed typical apoptotic features, nuclear condensation and nuclear fragmentation upon DAPI staining. Positive membranous staining for Annexin-V was also seen in these cells. Both caspase-8 and caspase-3 activities were up-regulated slightly. Pro-caspase-8 protein levels decreased slightly in both cells. Although the expression of Bcl-x_L was not influenced by Cpd 5, that of XIAP decreased in HLE cells. However, the pan-caspase inhibitor, zVAD, could not significantly prevent Cpd 5-induced apoptosis and Cpd 5 could not augment TRAIL-induced apoptosis. These results demonstrate that Cpd 5 induced apoptosis in human HCC cell lines, mainly independently of caspase activities. This may contribute to its highly potent cytotoxicity toward HCC cells. *Anti-Cancer Drugs* 16:837–844 © 2005 Lippincott Williams & Wilkins.

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Introduction

Dysregulation of apoptosis signaling is often associated with disease formation, such as autoimmune diseases [1] and cancer [2]. In hepatocarcinogenesis, failure of apoptosis is considered to be important for the survival of hepatocytes that are prone to genetic damage and cellular transformation through increased proliferation due to hepatocyte regeneration [3,4]. In fact, most hepatocellular carcinoma (HCC) cells show strong resistance to various stimuli that otherwise induce apoptosis [5]. Several cellular anti-apoptotic mechanisms such as reduced expression of Fas [4,6] or caspases [7], as well as expression of anti-apoptotic proteins, the Bcl-2 family [8,9] and inhibitors of apoptosis families [10,11] or receptor-mediated survival signals [12,13], are known to contribute to resistance against immunologic cytotoxicity in human HCC cells.

Vitamin K is usually identified as a critical factor in blood coagulation, and inhibition of cancerous cell growth *in vivo* and *in vitro* by vitamin K has been also observed [14–22]. The various forms of vitamin K include vitamin K_1 (phylloquinone), which is found in vegetables and vitamin K_2 (menaquinone), which is produced by natural bacteria in the intestine. Other K vitamins, such as vitamin K_3 (menadione), are synthetic vitamin K congeners. Although vitamin K_1, K_2 and K_3 have growth inhibitory effects, these effects are not strong. Recently, a novel K vitamin analog, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone [compound 5 (Cpd 5)], has been found to be a more potent growth inhibitor than natural or many synthetic K vitamins in HCC cells and normal rat hepatocytes [23,24]. From its chemistry, Cpd 5, which has a short side-chain containing a thioethanol group, was suggested to act through arylation of cellular thiols or thiol-dependent proteins by an addition–elimination mechanism [25]. It has been proposed that Cpd 5 inhibits protein tyrosine phosphatases (PTPases) through arylation of their critical cysteines, which are known to play an essential role in the catalytic process of PTPases [26]. The growth inhibitory effect of vitamin K_3 has been shown to result from both sulfhydryl arylation and oxidative stress mediated by redox cycling [27,28]. The growth-inhibitory effect of Cpd 5 is due to inactivation of

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cellular PTPase via sulfhydryl arylation of its catalytic cysteines rather than oxidative stress [30,31].

Mitogen-activated protein kinases (MAPKs) are important components of signaling transduction pathways. The extracellular signal-related kinase (ERK) signaling pathway is tightly associated with growth inhibition of HCC cells [32,33] and in rat hepatocytes caspase-3 is activated by Cpd 5 after ERK phosphorylation [34]. However, the anti-tumor mechanisms of Cpd 5 have not been completely elucidated. Therefore, we investigated the effect of Cpd 5 on human HCC cell lines and evaluated the apoptotic mechanism involved in the caspase-mediated pathway.

Materials and methods

Materials

The Cpd 5 used in this study was synthesized by B. I. Carr. Cpd 5 was synthesized as described previously [23].

Cell lines

The human HCC cell lines, SK-Hep1 and HepG2, were purchased from ATCC (Rockville, Maryland, USA). The HCC cell line, HLE (JCRB 0404), was purchased from the Health Science Research Resources Bank (Osaka, Japan). All HCC cells were cultured in DMEM (Dainippon, Osaka, Japan) at 37°C. All media were supplemented with 1% penicillin/streptomycin (Gibco/BRL, New York, USA) and 10% heat-inactivated fetal calf serum (Gibco/BRL).

Detection of caspases and apoptosis-related protein by Western blotting

Expression of caspase-8 and caspase-3 in HCC cell lines and Bcl-x and XIAP in HCC cell lines was analyzed by Western blotting. Briefly, cells were harvested and lysed in lysis buffer (50 mmol/l Tris–HCl, pH 8, 150 mmol/l NaCl, 5 mmol/l EDTA, 1% NP-40 and 1 mmol/l PMSF) on ice. After centrifugation, supernatants were collected and the protein content was measured using a Bio-Rad (Hercules, California, USA) protein assay kit. Equal amounts of protein from each extract were separated by 15% SDS–PAGE and transferred onto nitrocellulose membranes (Toyo Roshi, Tokyo, Japan) using the Bio-Rad electrotransfer system. Blots were blocked by incubating in 5% milk with Tris–HCl (pH 7.5) and 0.1% Tween 20 for 2 h at room temperature and probed overnight at 4°C with rabbit anti-caspase-8 (FLICE) polyclonal antibody (Upstate Biotechnology, Lake Placid, New York, USA), goat anti-caspase-3 (CPP32) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA), rabbit anti-Bcl-x_L polyclonal antibody (Transduction, Lexington, Kentucky, USA) or mouse anti-XIAP (Transduction). Antibodies were diluted 1:1000 with 5% milk in Tris–HCl (pH 7.5) and 0.1% Tween 20. The immunoblots were then probed with horseradish peroxidase-conjugated anti-rabbit IgG, horseradish peroxidase-conjugated anti-goat IgG or horseradish peroxidase-conjugated anti-mouse IgG (1:2000 diluted with 5% milk in Tris–HCl, pH 7.5). After the final wash, the signal was detected with an ECL kit (Amersham Pharmacia Biotech, Little Chalfont, UK).

Detection of apoptosis

A total of 2×10^5 HLE cells and SK-Hep1 cells were cultured in 35-mm culture dishes for 24 h, followed by the addition of 40 μM Cpd5. After incubation for 24 h, cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St Louis, Missouri, USA) and observed under a fluorescence microscope (Zeiss, Göttingen, Germany). To identify early apoptotic changes, after incubation with Cpd 5 for 9 h, the cell surface was stained with FITC-conjugated anti-Annexin-V antibody (Boehringer Mannheim, Mannheim, Germany) and observed under a fluorescence microscope. To assess the viability of HCC cells, the MTT assay was performed. The HCC cells were plated at a density of 5×10^3 cells/well in 96-well flat-bottom microtiter plates (Corning Glass Works, Corning, New York, USA) and each plate was incubated for 24 h at 37°C in 5% CO_2. Next, 50 μl of drug solution (Cpd 5 0–80 μM) was added to each well and the plates were incubated for 48 h. Then, the live cell count was assayed with a Cell Titer 96 Assay Kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The absorbance of each well was measured with a microtiter plate reader (Bio-Rad, Tokyo, Japan) at 570 nm.

Caspase activity assay

Protease activity was measured using tetrapeptide *p*-nitroanilide substrates in a colorimetric protease assay (MBL, Nagoya, Japan). The assays were performed in 96-well plates by incubating 50 μl of cell lysates with 50 μl of reaction buffer containing 200 μmol/l of peptide substrate. The assays included Ile-Glu-Thr-Asp-pNA (IETD-pNA, FLICE) or Asp-Glu-Val-Asp-pNA (DEVD-pNA, CPP32). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Bio-Rad).

*z*VAD inhibition

An MTT assay was performed to assess the influence of Cpd 5 after zVAD inhibition. The HCC cells were plated at a density of 5×10^3 cells/well in 96-well flat-bottom microtiter plates and each plate was incubated for 24 h at 37°C in 5% CO_2. Caspase inhibitor zVAD (z-Val-Ala-Asp fluoromethylketone; Calbiochem, California, USA) was pre-incubated for 3 h, 50 μl of drug solution (Cpd 5 0–80 μM) was added to each well and the plates were incubated for 24 h. Then, the live-cell count was assayed by using a Cell Titer 96 Assay Kit according to the manufacturer's instructions. The absorbance of each well was measured on a microtiter plate reader at 570 nm.

Influence of Cpd 5 on TRAIL-induced apoptosis in HCC cell lines

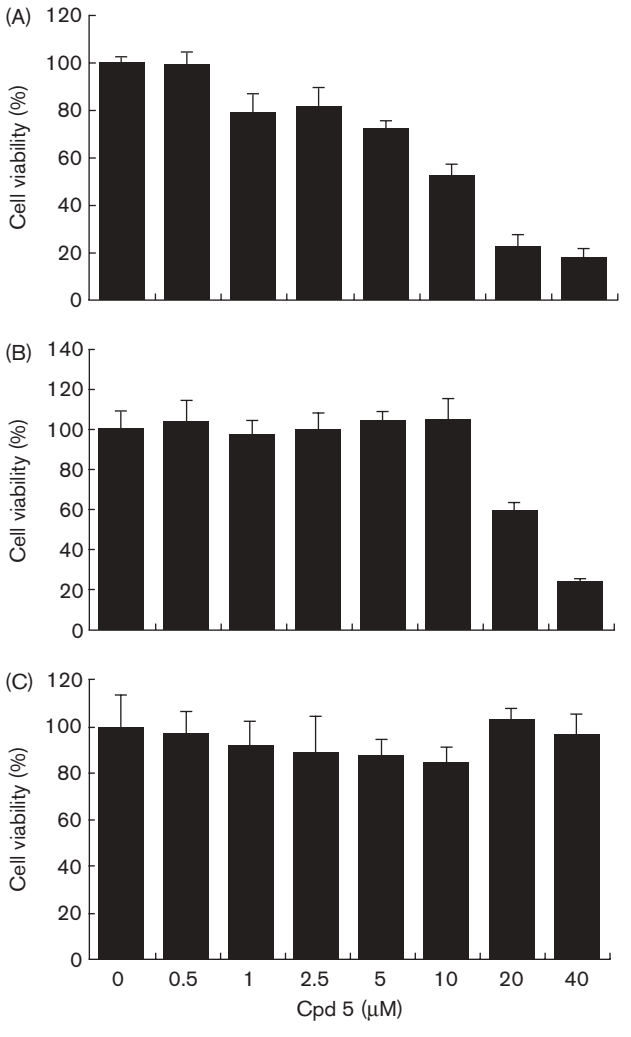
An MTT assay was performed to assess the influence of Cpd 5 on TRAIL-induced apoptosis in HCC cell lines. The HCC cells were plated at a density of 5×10^3 cells/well in 96-well flat-bottom microtiter plates and each plate was incubated for 24 h at 37°C in 5% CO_2. HCC cells were incubated with various concentrations of recombinant human TRAIL (R & D System, Minneapolis, MN) in the absence or presence of Cpd 5. The MTT assay method is described above.

Results

Cpd 5 induced apoptosis in HCC cells

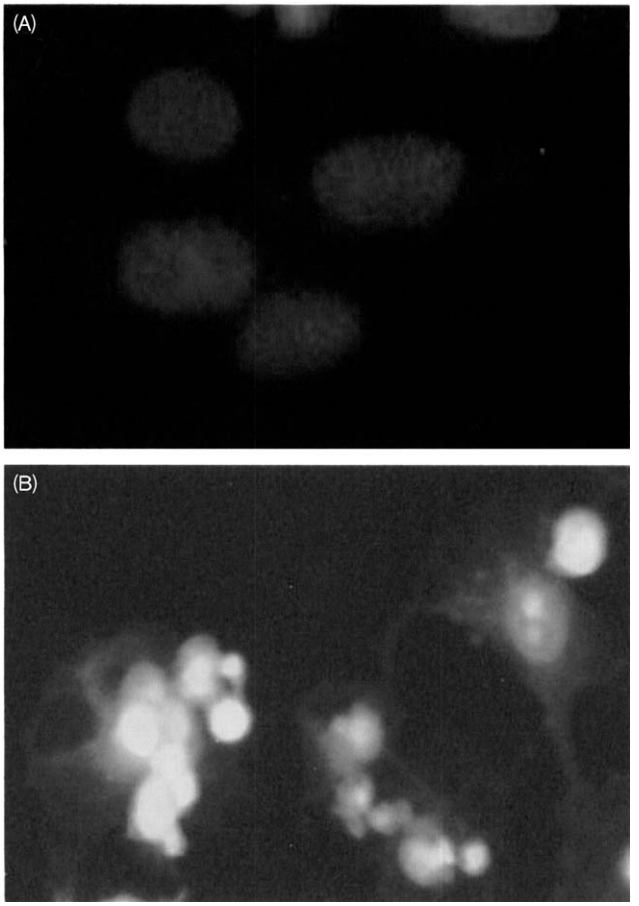
To investigate the change in viability of HCC cells in response to Cpd 5, HCC cells were incubated with various concentrations of Cpd 5 for 48 h. Cell viability was assessed by the MTT assay. Although HepG2 cells were not influenced by Cpd 5, Cpd 5 decreased cell viability in the other two HCC cells (HLE and SK-Hep1) in a concentration-dependent manner (Fig. 1). In order to investigate whether Cpd 5 induces apoptosis in HCC cells or not, we assessed DAPI staining 24 h after Cpd 5 (40 μM) treatment and Annexin-V membrane staining 9 h after Cpd 5 (40 μM) treatment of HLE and SK-Hep1 cells. Although apoptosis was not induced in either untreated cell line (Fig. 2A), Cpd 5-treated cells showed typical apoptotic features including nuclear condensation and nuclear fragmentation with DAPI staining (Fig. 2B). The same change was seen in HLE cells (data not shown). Positive membranous staining for Annexin-V, which is indicative of early apoptosis, was also clearly seen in both cell lines (Fig. 3B and D).

Fig. 1



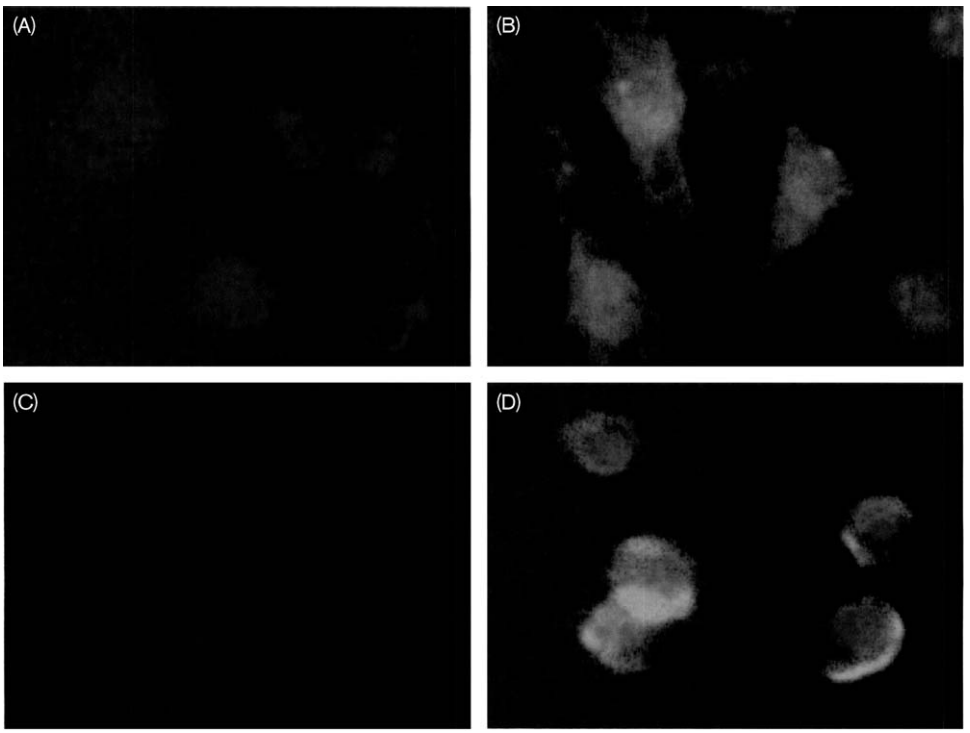
Effect of Cpd 5 on HCC cell lines: (A) HLE, (B) SK-Hep1 and (C) HepG2. HCC cells were incubated with various concentrations of Cpd 5 for 48 h. Cell viability was assessed by the MTT assay. The data shown are the means ± SD of eight independent experiments.

Fig. 2



Cpd 5-induced apoptosis in SK-Hep1 cells. Cells were cultured in the (A) absence or (B) presence of 40 μM Cpd 5 for 24 h. Cell nuclei were stained with DAPI. Note the typical apoptotic features of nuclear condensation and nuclear fragmentation.

Fig. 3



Cpd 5-induced apoptosis in HCC cell lines. (A and B) Cells were cultured in the (A) absence or (B) presence of 40 μM Cpd 5 for 24 h in HLE cells. (C and D) Cells were cultured in the (C) absence or (D) presence of 40 μM Cpd 5 for 24 h in SK-Hep1 cells. Cells were stained with FITC-conjugated anti-Annexin-V antibody to indicate early stages of apoptosis.

Effect of Cpd 5 on caspase activity in HCC cells

Apoptosis has been reported to be mediated chiefly by specific cysteine proteases and caspases, which are unique in cleaving substrates specifically after aspartate residues [35,36]. Apoptotic cell death usually results from the action of caspases and since caspase activation is essential for the apoptotic response, we examined caspase activation in Cpd 5-treated HCC cell lines. In order to assess the potential roles of caspase family members in Cpd 5-mediated HCC cell apoptosis, caspase-8 and caspase-3 were analyzed by colorimetric assay and Western blotting. Colorimetric assay was performed using cells incubated in the absence or presence of 40 μM Cpd 5 for 24 h. Neither caspase-8 nor caspase-3 activities were up-regulated in the HepG2 cell line. Their activities were slightly up-regulated in HLE ;and SK-Hep1 cell lines when treated with Cpd 5, but the increase was not significant (Fig. 4). Western blotting was performed using HLE and SK-Hep1 cell lines at 24 h after Cpd 5 treatment. Although pro-caspase-3 protein levels did not decrease, those of pro-caspase-8 decreased slightly in both cell lines at a concentration of 40 μM Cpd 5 (Fig. 5).

Changes in apoptosis-related proteins due to Cpd 5

Both Bcl-x_L and XIAP play a major role in controlling apoptotic pathways. To determine the expression of apoptosis-related proteins, we analyzed the expression of Bcl-x_L [37], which is an apoptosis inhibitory protein, and XIAP [38–40], which is a direct inhibitor of cell-death proteases, by Western blotting. The expression of XIAP was not down-regulated in SK-Hep1 cells, but was down-regulated in HLE cells in a concentration-dependent manner (Fig. 5). The expression of Bcl-x_L was not influenced by Cpd 5 in either cell line (Fig. 5).

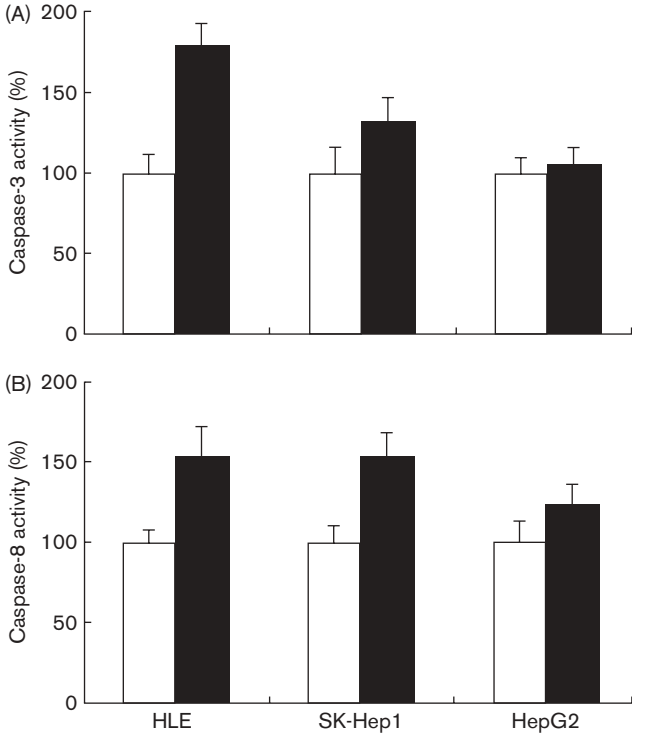
Cpd 5-induced apoptosis was not inhibited by zVAD

In order to determine whether or not the caspase pathway is related to the apoptosis of HCC cells induced by Cpd 5, we examined the changes in viability of HCC cells treated with Cpd 5 and with or without zVAD, a strong pan-caspase inhibitor. However, zVAD did not significantly prevent Cpd 5-induced apoptosis in either cell line (Fig. 6).

Cpd 5 could not enhance TRAIL-induced apoptosis in HCC cell lines

It is well known that TRAIL induces apoptosis in HCC cell lines via the caspase pathway [41]. To examine the relationship between Cpd 5 and TRAIL-induced apoptosis in HCC cell lines, HCC cells were incubated with various concentrations of TRAIL in the absence or presence of a subtoxic level of Cpd 5 (10 μM) for 24 h.

Fig. 4



Caspase-3 and caspase-8 colorimetric assay. The data are presented as OD 405 nm for HLE, SK-Hep1 and HepG2 cells incubated in the absence (open columns) or presence of 40 μM Cpd 5 (filled columns) for 24 h. The data shown are the means ± SD of four independent experiments.

Although cell viability decreased in response to TRAIL in a dose-dependent manner (Fig. 7, control) in both cell lines, the TRAIL Cpd 5 combination decreased cell viability only additively, not synergistically (Fig. 7). These results indicate that Cpd 5 did not affect the TRAIL-induced apoptosis pathway in HCC cell lines.

Discussion

HCC is one of the more common malignant tumors. To prevent development and recurrence of HCC, growth inhibition is very important. The vitamin K family has great potential for efficacy in this context. Clinically, oral administration of vitamin K_2 prevents portal venous invasion in HCC patients with high des-γ-carboxy prothrombin levels [42], and oral administration of vitamin K_2 decreases the cumulative incidence of recurrent HCC and improves cumulative survival rate [43]. In addition, oral administration of vitamin K_2 is reported to reduce the recurrence of HCC in women with viral cirrhosis [44].

Cpd 5 is more potent than natural vitamin K. Growth inhibition of HCC by Cpd 5 *in vitro* and *in vivo* has been previously reported [23,31,32,45]. One of the inhibitory mechanisms of Cpd 5 is related to its induction of persistent ERK phosphorylation [33,46,47], which has been shown to induce apoptosis in rat hepatocytes [34]. We present evidence here that Cpd 5 induces human hepatoma cell apoptosis. The typical apoptotic features of nuclear condensation and nuclear fragmentation were observed upon DAPI staining, and positive Annexin-V staining was observed in HLE and SK-Hep1 cell lines. Both HLE and SK-Hep1 cell lines containing mutant p53 exhibited apoptosis induced by Cpd 5, although HepG2 containing wild-type p53 did not. These results indicated that Cpd 5-mediated apoptosis might be related to p53 status.

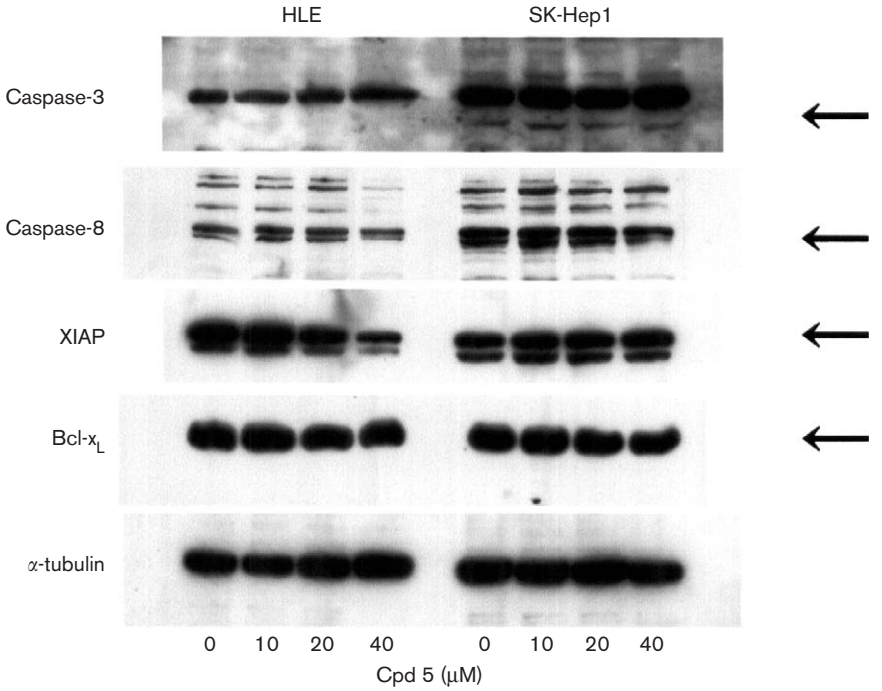
It has been reported that Cpd 5-mediated growth inhibition in HCC cell lines and hepatocytes is related to ERK. MAPK pathways are one of the most important components of signaling transduction pathways and the ERK signaling pathway is very closely related to inhibition of tumor cell growth.

In this study, we analyzed the effect of Cpd 5 on human HCC cell lines with special reference to apoptosis involving the caspase-mediated pathway. We examined caspase-8 and caspase-3 activity using a colorimetric assay and Western blotting. Although the activities of caspase-8 and caspase-3 were slightly up-regulated in HLE and SK-Hep1 cells in the colorimetric assay, the changes were not significant. In HepG2 cells, Cpd 5 had no effect. Pro-caspase-3 and pro-caspase-8 protein levels also did not change significantly. These results suggested that Cpd 5 had little effect on caspase activity.

We also analyzed changes in apoptosis-related proteins due to Cpd 5. In rat hepatocytes, Cpd 5 induces ERK phosphorylation and subsequently activates the apoptosis cascade, including altered expression of Bcl-2/Bax, activation of caspase-3 and DNA fragmentation [34]. Both Bcl-x_L and XIAP play a major role in controlling caspase-mediated apoptotic pathways. Bcl-x_L, which is an apoptosis inhibitory protein and which inhibits the mitochondrial apoptotic pathway [37], and XIAP, which is a direct inhibitor of caspases [38–40], were analyzed by Western blotting. Although the expression of Bcl-x_L was not influenced by Cpd 5 in either cell line, the expression of XIAP was down-regulated in the HLE cell line in a concentration-dependent manner.

In order to elucidate the impact of caspase activity on Cpd 5-induced apoptosis in HCC cells, we investigated caspase inhibition. The change in HCC cell viability in response to Cpd 5 was not suppressed by zVAD, a strong pan-caspase inhibitor. These results indicate that the caspase-mediated pathway was not fully involved in Cpd

Fig. 5



Expression of caspase-3 and caspase-8 in human HCC cell lines and changes in apoptosis-related proteins in response to Cpd 5 in human HCC cell lines. Cellular lysates were separated by SDS–PAGE and transferred to nitrocellulose. Caspases, XIAP and Bcl-x_L levels were detected by Western blotting.

5-mediated apoptosis, suggesting the existence of a non-caspase-dependent pathway.

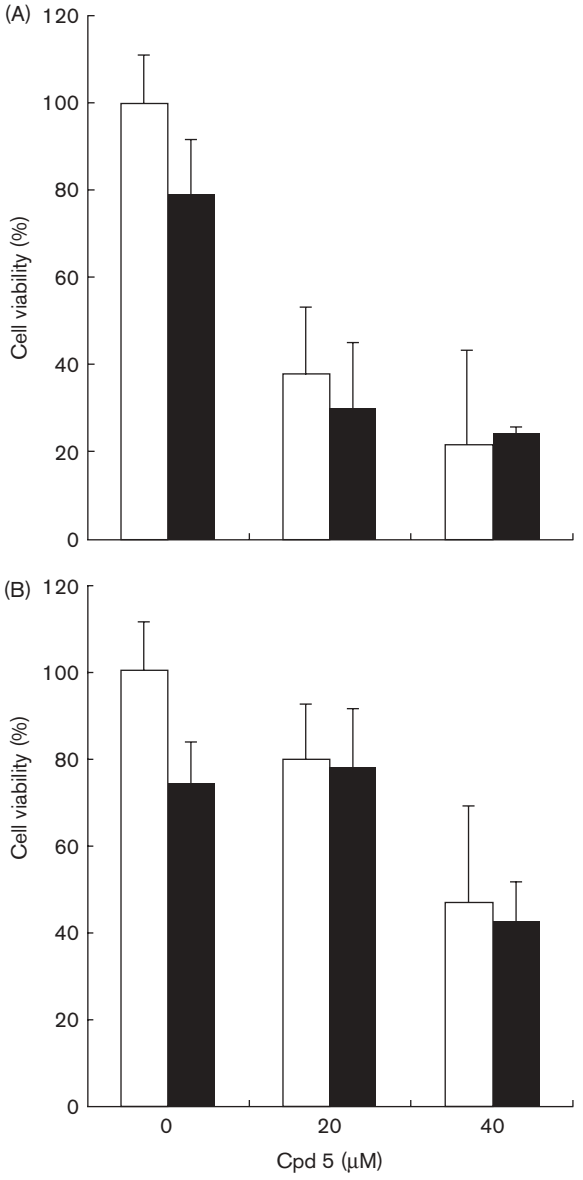
Additionally, we investigated the relationship between the Cpd 5-mediated apoptosis pathway and the TRAIL-induced apoptosis pathway [48,49]. The TRAIL-induced apoptosis pathway involves increases in caspase activity. If Cpd 5-mediated apoptosis in HCC cell lines was mediated by the caspase-mediated apoptotic pathways, Cpd 5 would have enhanced TRAIL-induced apoptosis in HCC cell lines. In general, TRAIL-induced apoptosis dramatically increases in combination with subtoxic levels of chemotherapeutic agents such as actinomycin D, doxorubicin and camptothecin [41]. However, in the present study, Cpd 5-mediated apoptosis was not influenced by subtoxic levels of these chemotherapeutic agents (data not shown). These results indicate that the caspase-mediated pathway was not involved in Cpd 5-mediated apoptosis in HCC cells.

Two major pathways have been described that regulate apoptosis: the intrinsic pathway, in which mitochondria play a central role, and the extrinsic pathway, in which cell plasma membrane receptors act as the starting point of the apoptotic process [50]. In both the intrinsic and extrinsic pathways, activation of caspases plays an indispensable role [51]. Recently non-caspase-dependant apoptosis has also been demonstrated. Apoptosis-inducing factor (AIF) is located in the mitochondrial intermembrane space, and once AIF is released from this location, it translocates to the nucleus and induces apoptosis [52]. Non-caspase proteases [53] including cathepsins, calpains, granzymes and endonuclease G [54] can induce apoptosis either independently or cooperatively with caspases. It has also been reported that granzyme B induces apoptosis independently of caspase activation [55]. Furthermore, lidamycin-induced apoptosis is caused directly by DNA cleavage at an early stage of the apoptotic process and is thus a non-caspase-mediated apoptotic pathway [56].

In our experiment, HCC cells containing mutant p53 are sensitive to Cpd 5-induced apoptosis and HCC cells containing wild-type p53 are not sensitive to Cpd 5. These results indicated that Cpd 5 directly or indirectly interacts with nuclear molecules, since p53 plays an important role in cell cycle regulation and apoptosis via interacting nuclear proteins.

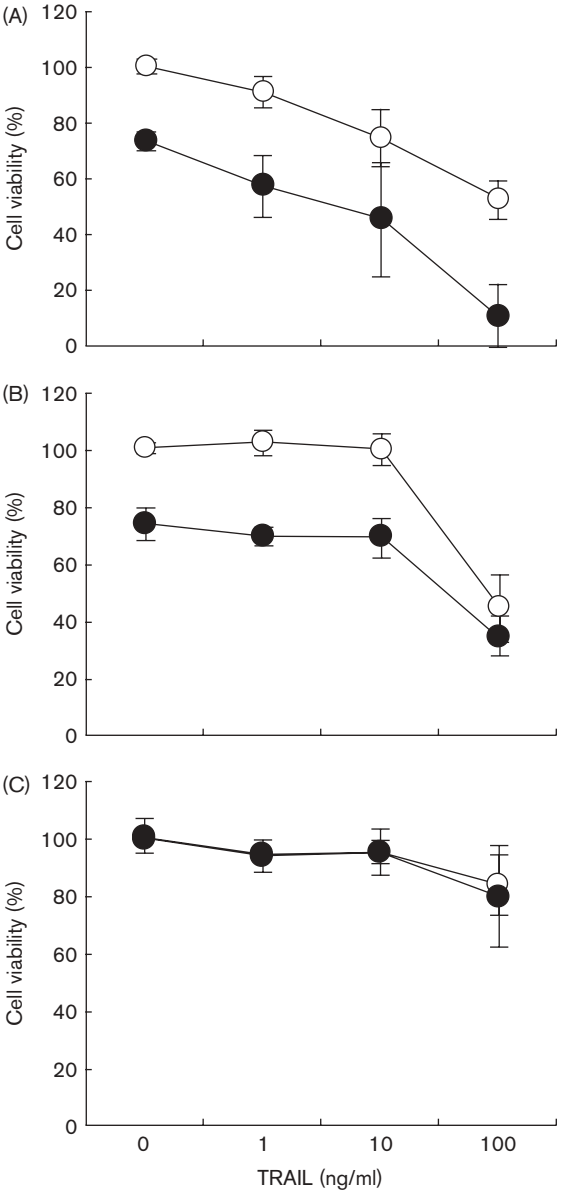
In conclusion, we present evidence here that Cpd 5, a K vitamin analog, induces human hepatoma cell apoptosis. The Cpd 5-mediated apoptosis in HCC cell lines mainly

Fig. 6



Influence of zVAD on Cpd 5-mediated human HCC cells apoptosis: (A) HLE and (B) SK-Hep1. HCC cells were incubated with various concentrations in the absence (open columns) or presence (filled columns) of 40 μM Cpd 5 for 24 h. Cell viability was assessed by the MTT assay. The data shown are the means ± SD of eight independent experiments.

Fig. 7



Influence of Cpd 5 on the TRAIL-induced apoptosis pathway in HCC cell lines (A) HLE, (B) SK-Hep1 and (C) HepG2. HCC cells were incubated with various concentrations of TRAIL in the absence (open circles) or presence (filled circles) of a subtoxic level of Cpd 5 (10 μM) for 24 h. Although cell viability decreased in response to TRAIL in a dose-dependent manner (control) in all cell lines, TRAIL in combination with the subtoxic level of Cpd 5 decreased cell viability only additively, not synergistically. Cell viability was assessed by the MTT assay. The data shown are the means ± SD of eight independent experiments.

involves a non-caspase apoptotic pathway. Further study will be needed to elucidate the anti-tumor mechanisms of vitamin K on tumor cells and establish Vitamin K-based anti-cancer therapy.

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