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Chemopreventive allylthiopyridazine derivatives induce apoptosis in SK-Hep-1 hepatocarcinoma cells through a caspase-3-dependent mechanism

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Abstract

Dietary organosulphur compounds including diallylsulphide, a component of garlic oil, were shown to inhibit the proliferation of tumour cells. Since hepatocellular carcinoma is one of the most lethal malignancies and there is no effective preventive measure to date, we wished to pursue the chemopreventive potential of the synthetic allylthiopyridazine derivatives (K compounds) on hepatocarcinoma cells. Here, we report that the K compounds efficiently inhibited SK-Hep-1 cell proliferation through induction of apoptosis. Increased chain length at the 3-position of allylthiopyridazine ring improved the potency of growth inhibition. K compounds downregulated Bcl-2, while Bax remained unchanged, reducing the ratio of Bcl-2 to Bax. We also provide evidence that the K compound-induced apoptosis involves cytochrome c release and caspase-3 activation. These results suggest that the allylthiopyridazine derivatives, especially 3-propoxy-6-allylthiopyridazine, induce apoptosis in SK-Hep-1 cells through a caspase-3-dependent mechanism, which may contribute to the chemopreventive function for hepatocellular carcinoma.

Keywords: Allylthiopyridazine; Hepatocarcinoma; Apoptosis; Bcl-2; Cytochrome c; Caspase-3

1. Introduction

Dietary organosulphur compounds including diallylsulphide, a component of garlic oil, have been shown to inhibit the proliferation of tumour cells [1–3] and to suppress chemically-induced carcinogenesis in various organs of experimental animals [4–7]. Some synthetic sulphur-containing compounds including oltipraz [8] and sulindac [9,10] have been shown to exert chemopreventive activities in experimental carcinogenesis. In spite of its early discovery in 1886, pyridazine has not gained much attention compared with the structurally similar compounds, pyrimidine and pyrazine, which are commonly found in nature. Recently, efforts have been made to reveal the biological and pharmacological activities of pyridazine derivatives. It has been reported that pyridazine and its derivatives show various activities including generation of reactive oxygen species [11], induction of hepatic microsomal enzymes [12], and regression of tumours [13].

Identifying and developing pharmaceutical agents that can selectively modulate apoptotic pathways may provide an effective tactic for the prevention and the treatment of cancer [14]. Mounting evidence suggests that activation of caspases trigger the apoptotic process in various cells [15]. Caspase-3 has been shown to play an essential role in apoptosis induced by a variety of stimuli [16]. One recently characterised mechanism for pro-caspase-3 activation involves translocation of the respiratory chain protein, cytochrome c, from the mitochondria to the cytoplasm [17,18]. Cytochrome c forms a complex resulting in the activation of pro-caspase-9, which, in turn, cleaves and activates pro-caspase-3. Bcl-2, an anti-apoptotic oncoprotein, has been shown to act on mitochondria and prevent the release of cytochrome c and thus caspase activation [18,19].

In an attempt to develop novel hepatoprotective and/or chemopreventive agents, we synthesised a series of allylthiopyridazine derivatives designated as K compounds (Fig. 1). 3-Alkoxy-6-allylthiopyridazines, the

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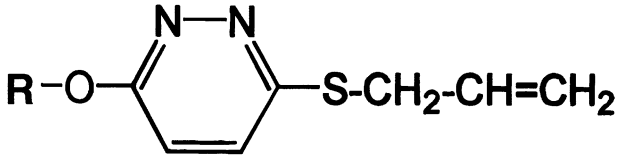
compounds having alkoxy residues in their *para* position, have been shown to exert hepatoprotective activities in experimental animals (manuscript in preparation). In order to extend our programme to pursue the chemopreventive potential of these compounds on hepatocellular carcinoma, we investigated the antiproliferative effect of the compounds in SK-Hep-1 hepatocarcinoma cells.

2. Materials and methods

2.1. Materials

The allylthiopyridazine derivatives were synthesised using pyridazine as a parent nucleus. 3-Alkoxy-6-chloropyridazines were obtained from 3,6-dichloropyridazine. 3-Alkoxy-6-allylthiopyridazines (K compounds) were then synthesised from halides and allylmercaptane. Shown in Fig. 1 are four allythiopyridazine derivatives used in this study: 3-methoxy-6-allylthiopyridazine (K6), 3-ethoxy-6-allylthiopyridazine (K16), 3-propoxy-6-allylthiopyridazine (K17) and 3-isopropoxy-6-allylthiopyridazine (K18). These compounds were selected based on the preliminary *in vivo* data in experimental animals. The synthetic compounds were proved to be highly pure as analysed by nuclear magnetic resonance (NMR) and Fourier transformed-infrared (FT-IR) (data not shown). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). A caspase-3 inhibitor, Ac-DEVD-CHO, was obtained from Takara Co. (Shiga, Japan).



Compounds	R
K6	- CH_3
K16	-C_2H_5
K17	- C_3H_7
K18	- CH(CH_3)(CH_3)

Fig. 1. Chemical structures of the allylthiopyridazine derivatives (K compounds).

4,6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma Chemical Co. (St Louis, MD, USA).

2.2. Cell line and culture conditions

SK-Hep-1 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were maintained at 37 °C in a humidified atmosphere at 95% air and 5% CO_2 in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin.

2.3. Growth inhibition

Cells (1×10^5) in a 48-well plate were cultured in the presence of various concentrations of K compounds (0.1 to 2.4 mM) for 48 h. Control cells were treated with dimethyl sulphoxide (DMSO) equal to the highest percentage of solvent used in the experimental conditions. Cells were counted and cell viability was determined by a trypan blue exclusion assay.

2.4. Nuclear staining

Cells were incubated in a six-well plate with or without 2 mM of K compounds for 5 h. Cells were fixed with 4% formaldehyde, dried, and stained with the DNA-specific fluorochrome DAPI. Following 30 min of incubation, cells were washed with phosphate-buffered saline (PBS), air dried, mounted with 90% (v/v) glycerol and observed under a fluorescence microscope (Olympus Optical Co., Japan).

2.5. DNA Fragmentation

Cells in a 100 mm dish were treated with 2 mM of K compounds for 48 h, trypsinised and collected with ice-cold PBS. Cell pellets were resuspended in isolation buffer (10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris–HCl, pH 8.0, 0.5% (w/v) sodium dodecyl sulphate (SDS), 0.5 mg/ml proteinase K) and incubated overnight at 50 °C. The lysate was centrifuged at 15 000 rpm for 15 min at 4 °C to separate the soluble fragmented DNA. Fragmented DNA was extracted and purified DNA was treated with 1 μg/ml RNase A for 1 h at 37 °C prior to electrophoresis on a 1.8% agarose gel containing ethidium bromide.

2.6. Immunoblot analysis

Equal amounts of protein extracts in lysis buffer (0.5% (v/v) Triton X-100, 0.15 M NaCl, 50 mM Tris–HCl, pH 7.4, 25 mM NaF, 20 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) were subjected to 12% SDS-polyacrylamide gel

electrophoresis (PAGE) analysis. The levels of Bcl-2 and Bax were detected using anti-Bcl-2 (1:1000) (mouse monoclonal purchased from DAKO, Denmark) and anti-Bax (1:2000) (rabbit polyclonal from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies. Anti-caspase-3 (CPP32) (1:2000) monoclonal antibody was purchased from Oncogene Science Inc. (Cambridge, MA, USA). Anti-β actin antibody (1:5000, Sigma Chemical Co.) was used to confirm the equal amount of protein loaded. The enhanced chemiluminescence (Amersham-Pharmacia, Buckinghamshire, UK) system was used for detection.

2.7. Determination of mitochondrial cytochrome c

Cells were harvested and washed once in PBS and then resuspended in three volumes of isolation buffer (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and protease inhibitor cocktail) in 250 mM sucrose. After the cells were homogenised in a glass Teflon homogeniser using 10 up-and-down strokes at 500 rpm, the homogenate was centrifuged at 10 000*g* for 15 min at 4 °C. The crude mitochondrial pellet was resuspended with lysis buffer (0.5% Triton X-100, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4) containing a protease inhibitor cocktail. The mitochondrial fraction (200 μg of protein) was subjected to 12% SDS-PAGE analysis. The levels of cytochrome c were detected using an anti-cytochrome c monoclonal antibody (1:2000, purchased from Zymed Laboratories Inc., San Francisco, CA, USA).

3. Results

3.1. Growth inhibition and morphological changes induced by the K compounds

A dose–response study was conducted to determine the antiproliferative activities of the K compounds. As shown in Fig. 2, treatment of the cells with the K compounds for 48 h inhibited SK-Hep-1 cell growth in a dose-dependent manner. IC_{50} values of K6 (3-methoxy-), K16 (3-ethoxy-), K17 (3-propoxy-) and K18 (3-isopropoxy-) for inhibiting SK-Hep-1 cell growth were 1.04, 0.97, 0.61 and 0.59 mM, respectively. For further investigation, we chose K17 as it had the highest growth-inhibitory potency and K16 with which the most effective *in vivo* hepatoprotective activity was observed (manuscript in preparation). Agents that affect malignant transformation often cause a dramatic morphological change in cells [20]. To test whether the K compounds affect the transformed phenotype of the hepatocellular carcinoma cells, we investigated the effect of K16 and K17 on the morphology of SK-Hep-1 cells. As shown in Fig. 3, treatment of K compounds prominently changed the morphology of the cells. Treatment with 1 mM of K compounds for 48 h produced characteristic elongated cells with filamentous protrusions.

3.2. Apoptosis was induced by the K compounds in SK-Hep-1 cells

To evaluate whether the growth-inhibitory effect of K compounds was associated with apoptosis, we examined nuclear morphological changes using a DNA-specific fluorescent dye, DAPI. When the SK-Hep-1 cells were treated with 2 mM of K16 or K17 for 5 h, they clearly exhibited condensed and fragmented nuclei, indicative of apoptotic cell morphology (Fig. 4a). To further confirm apoptotic cell death, we examined whether the K compounds induced internucleosomal degradation of DNA, a characteristic of apoptosis. As shown in Fig. 4b, treatment with 2 mM of K16 or K17 for 48 h induced nucleosomal DNA fragmentation in SK-Hep-1 cells, clearly demonstrating that the K compounds induced apoptosis in hepatocarcinoma cells.

3.3. K compounds reduced the ratio of Bcl-2 to Bax

In an attempt to unveil the molecular mechanisms underlying the K compounds-induced apoptosis of SK-Hep-1 cells, we measured the protein levels of two key apoptosis-linked gene products, Bcl-2 and Bax, which are known to regulate the cell death/survival in opposite manners. As shown in Fig. 5, expression of the anti-

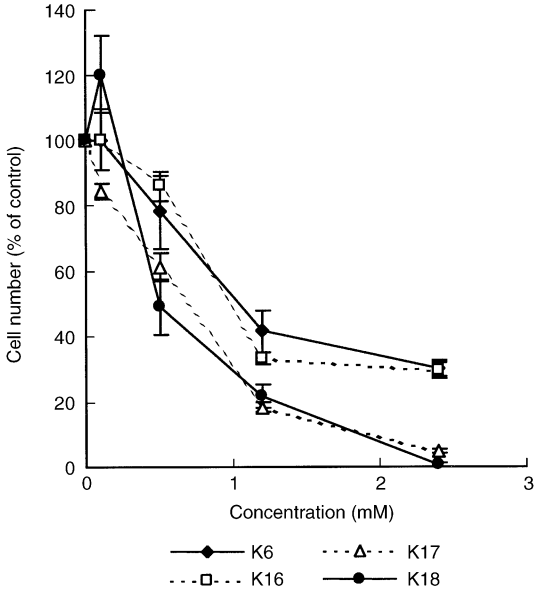
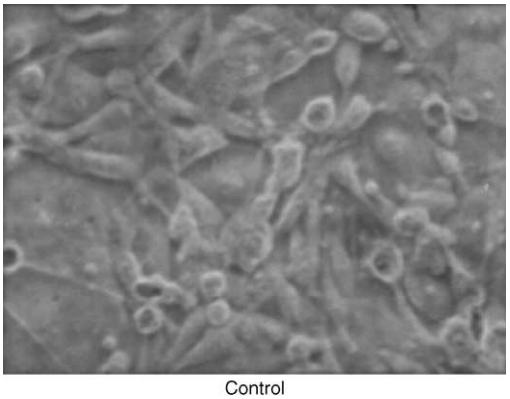
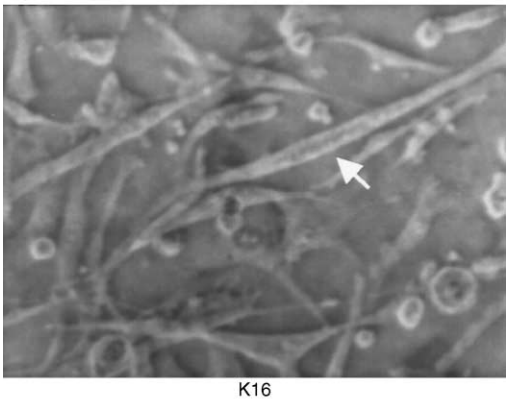


Fig. 2. K compounds inhibit the SK-Hep-1 cell growth in a dose-dependent manner. SK-Hep-1 cells (1×10^5) in a 48-well plate were treated with K compounds for 48 h and counted. The results presented are ±standard error of the mean (SEM) of triplicates.

apoptotic oncoprotein Bcl-2 was significantly decreased, whereas the death-promoting Bax expression remained unchanged during the apoptosis process in the SK-Hep-1 cells treated with K16 or K17 (2 mM) for 24 h. A stronger effect of decreasing Bcl-2 level was observed in the K17-treated cells. The ratios of Bcl-2 to Bax were decreased to 0.4 and 0.3 of control for the K16- and K17-treated cells, respectively. The results suggest that one mechanism by which the K compounds induce apoptotic cell death is to reduce the ratio of Bcl-2 to Bax.





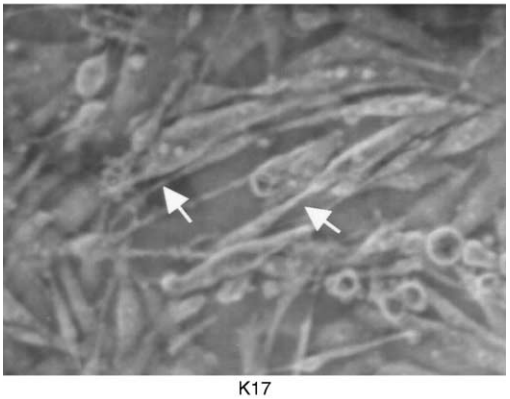


Fig. 3. K compounds induce morphological changes in the SK-Hep-1 cells. The SK-Hep-1 cells were treated with 1mM of K compounds for 48 h and the morphology of the cells was examined microscopically (100×). Filamentous protrusions in the treated cells are noted by arrows.

3.4. Mitochondrial cytochrome c was released during K compound-induced apoptosis

Bcl-2 has been shown to block cytochrome c release from the mitochondria and thereby inhibit caspase activation and the apoptotic process [18,19]. Therefore, we decided to measure the level of cytochrome c in mitochondria at different times after K compound treatment. Western blot analysis revealed a marked decrease in the mitochondrial cytochrome c level in K17-treated SK-Hep-1 cells detectable 36 h after treatment (Fig. 6). The mitochondrial fraction was probed for β-actin to confirm the equal loading on the gel.

3.5. Activation of caspase-3 by K compounds

Since it was shown that released cytochrome c plays a key role in the activation of caspase-3 [17], we then examined whether caspase-3 was activated during K compound-induced apoptosis. Activation of caspase-3 was determined by monitoring proteolysis of the 32-kDa procaspase-3 to 17- and 12-kDa fragments. Fig. 7a shows a time-dependent appearance of the 17-kDa fragment of the active enzyme following exposure to K17. The 17-kDa fragment was initially seen at 8 h post-treatment. In order to examine the functional role of caspase-3 in K compound-induced apoptosis, we pretreated the SK-Hep-1 cells with a specific inhibitor of caspase-3, Ac-DEVD-CHO [21], and then exposed them to K17. As shown in Fig. 7b, Ac-DEVD-CHO significantly reversed the K17-induced growth inhibition, suggesting that DEVDase (caspase-3) activity is critical to the K compound-induced apoptosis in SK-Hep-1 cells. Taken together, we show that allythiopyridazine derivatives induce apoptosis in SK-Hep-1 hepatocarcinoma cells through a caspase-3-dependent mechanism, which may contribute to the chemopreventive function of these agents for hepatocarcinoma.

4. Discussion

The major aim of this investigation was to evaluate the chemopreventive potential of the organosulphur compounds, allylthiopyridazine derivatives (K compounds), against the growth of SK-Hep-1 hepatocellular carcinoma cells. We provide evidence that the K compounds, especially 3-propoxy-6-allylthiopyridazine (K17), inhibited SK-Hep-1 hepatocarcinoma cell proliferation with the IC_{50} value of 0.93 mM. The K compounds showed more potent anti-proliferative activities compared with a structurally similar compound, 2-(allylthio) pyrazine, which has been reported to inhibit the growth of HL-60 cells with the IC_{50} value of approximately 2 mM [22]. Increased chain length at the

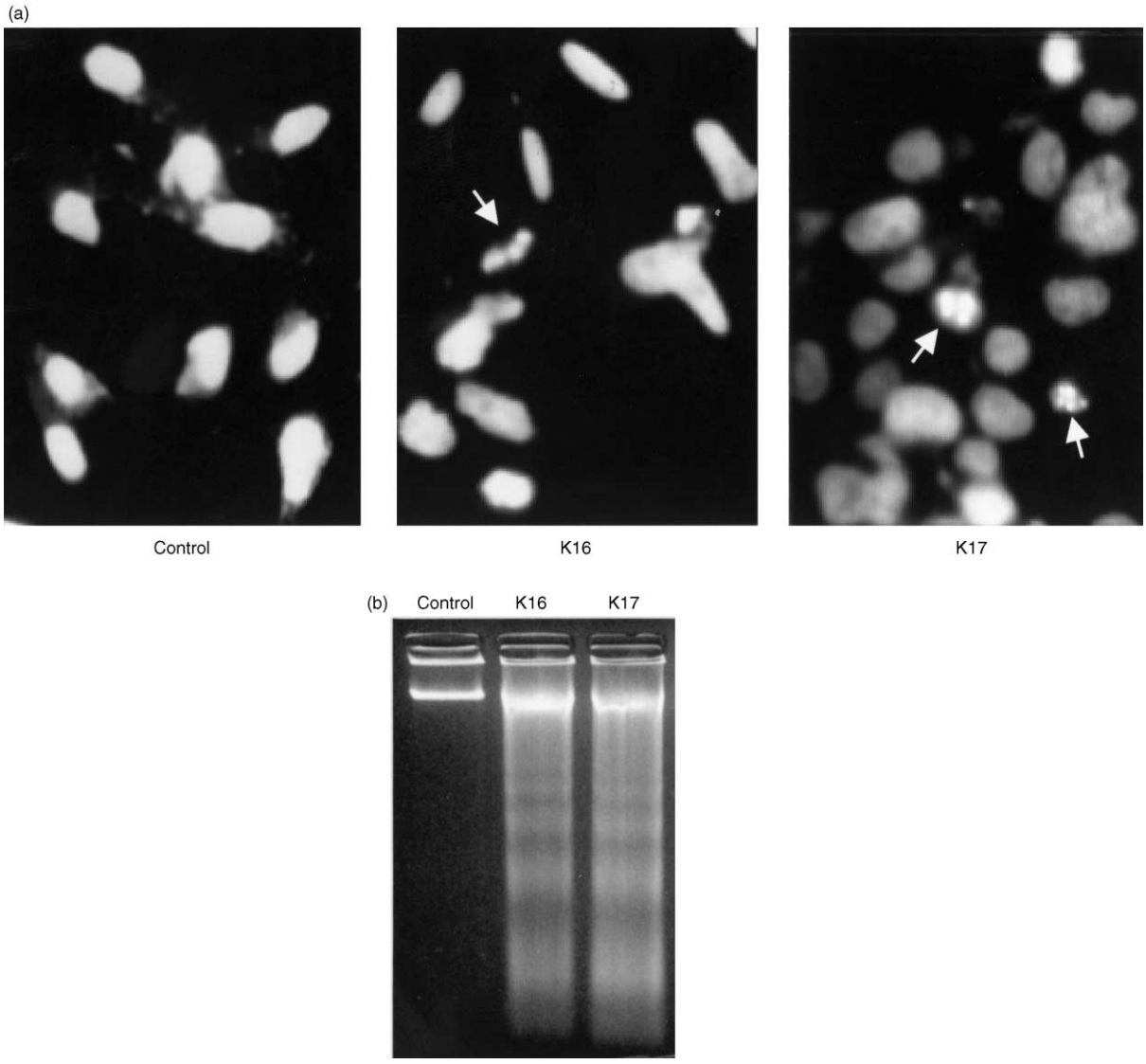


Fig. 4. K compounds induce apoptosis in SK-Hep-1 cells. (a) Nuclear morphological analysis. Cells were treated with 2 mM of K16 or K17 for 5 h and stained with 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Stained cells were examined by fluorescence microscopy. Potential apoptotic nuclei are indicated by arrows. (b) Internucleosomal DNA fragmentation. DNA was extracted from cells treated with 2 mM of K16 or K17 for 48 h and analysed by agarose gel electrophoresis.

3-position of allylthiopyridazine ring improved the potency of growth inhibition (3-propoxy > 3-isopropoxy > 3-ethoxy > 3-methoxy derivatives). High potency seen with either K17 (3-propoxy-) or K18 (3-isopropoxy-) might possibly be due to a better penetration into the cell. Preliminary *in vivo* safety data (acute oral toxicity) showed that no abnormalities were observed in rodents treated with the compounds at a dose of 1.3 mmol/kg/day.

The present study reveals that the inhibitory effect of the K compounds on SK-Hep-1 cell growth is mainly due to the induction of apoptosis. A growing body of evidence suggests that synthetic sulphur-containing compounds such as sulindac and exisulind induce apoptosis in various tumour cell lines [5,10]. The efficacy of the K compounds was lower than that of sulindac which induced cellular apoptosis at a concentration of 100 μM in hepatocellular carcinoma cell lines [10].

Bax counteracts the anti-apoptotic effects of Bcl-2 by forming a heterodimer with Bcl-2 [23]. The ratio of Bcl-2 to Bax, rather than the levels of the individual proteins, is considered to be critical in determining the survival/death of cells [24,25]. Our results show that K compounds greatly reduced the ratio of Bcl-2 to Bax which may trigger apoptosis in the SK-Hep-1 cells.

Translocation of cytochrome c from the mitochondria to the cytoplasm has recently been shown to be one of the key events in triggering the activation of caspase-3 during the induction of apoptosis by a variety of different agents [18]. In this study, we show that the K compound induced the release of cytochrome c from the mitochondria and activation of caspase-3 in SK-Hep-1

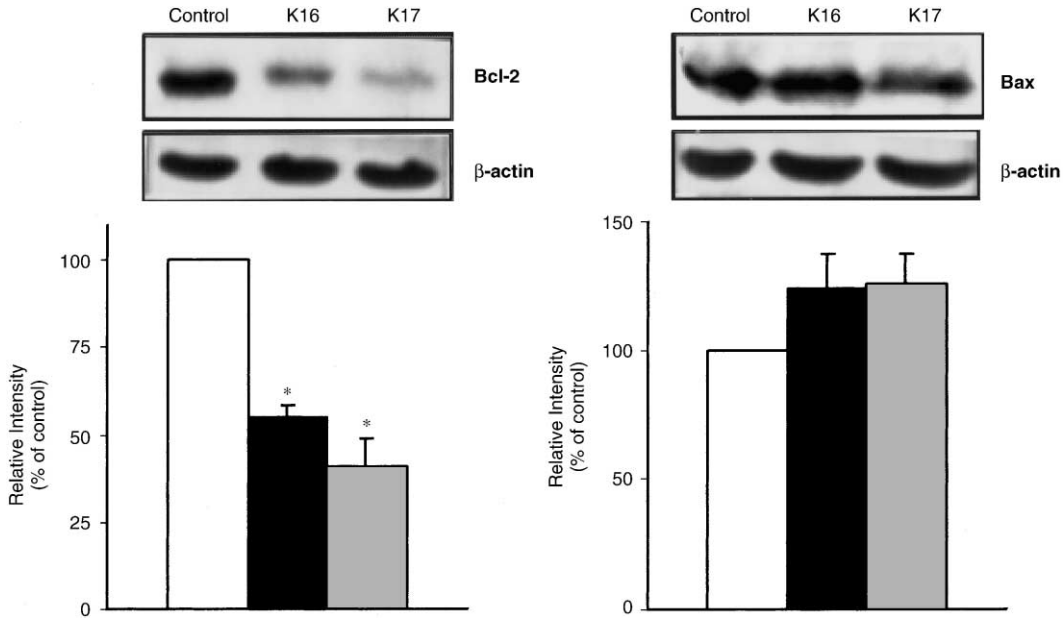


Fig. 5. K compounds downregulate Bcl-2 in SK-Hep-1 cells. Western blot analysis was performed on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with lysates (30 μg protein) from cells treated with 2 mM of K16 or K17 for 24h. Relative band intensities were determined by quantitation of each band and the bars presented are ±standard error of the mean (SEM) of data from three independent experiments. *Statistically different from control at $P<0.05$.

cells at a 2 mM concentration which caused a prominent decrease of Bcl-2. It is suggested that downregulation of Bcl-2 may accelerate cytochrome c release and caspase-3 activation during K compound-induced apoptosis of SK-Hep-1 cells. The time course of the appearance of the 17 kDa fragment of the active capsase-3 (Fig. 7) does not correlate well with the cytochrome c release from the mitochondria (Fig. 6). A possible explanation would be that at earlier time points, a small amount of cytochrome c released from mitochondria, not detectable by Western blot analysis, may have efficiently functioned as an activator of caspase-9, resulting in the caspase-3 activation. Alternatively, caspase-8-mediated activation of procaspase-3, independent of cytochrome c, might be the mechanism involved since pro-caspase-3 can also be directly activated by caspase-8 [26]. Further studies need to be performed to elucidate the molecular mechanisms for the K compound-induced apoptosis.

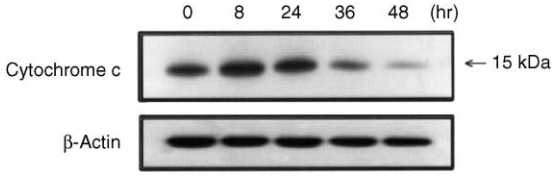


Fig. 6. Mitochondrial cytochrome c is released by treatment of cells with the K compound. The mitochondrial fraction (200 μg) was prepared from SK-Hep-1 cells treated with 2 mM K17 for the indicated times and subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis with an anti-cytochrome c antibody.

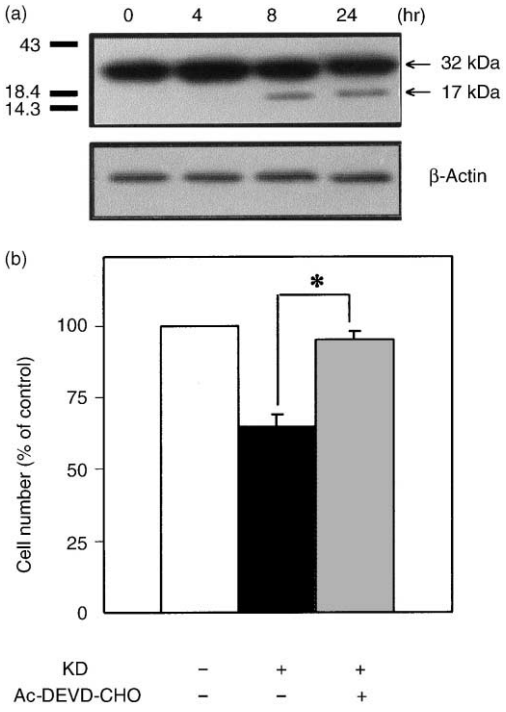


Fig. 7. Caspase-3 is activated by a K compound. (a) SK-Hep-1 cells were treated with 2 mM of K17 for the indicated times. Cell lysates (80 μg) were subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis was performed with an anti-CPP32 antibody. Shown left are molecular weight markers (kDa). (b) Cells (1×10^5) were preincubated with 100 μM Ac-DEVD-CHO (a caspase-3 inhibitor) for 2 h before being treated with 0.5 mM of K17 for 48 h and counted. The results presented are ±standard error of the mean (SEM) of triplicates. *Statistically different at $P<0.05$.

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